The Effects of Strain Variation on Respiratory Syncytial Virus Infection and Immunity

Thesis

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The effects of strain variation on Respiratory Syncytial Virus infection and immunity

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Abstract

Introduction: The transmission of RSV in the human population is characterised by seasonal epidemics displaying cyclic alternation in dominance of the two antigenic groups A and B. The mechanisms that underlie this population-level competition between RSV A and B, and heterogeneity in transmission fitness of RSV genotypes are not clearly defined. The dynamics of neutralising antibody response following birth and primary infection as well as pro- and anti- inflammatory cytokines/chemokines following natural infection in infants, especially in relation to RSV antigenic variation, have not been well characterised.

Methods: Neutralising antibodies were measured in the acute and convalescent sera of infants with RSV-associated pneumonia as well as from a birth cohort. Additionally, 10 cytokines/chemokines were measured in nasal secretions obtained from infants recruited within the household setting and from whom samples were obtained twice weekly during an RSV epidemic.

Results: The proportion of RSV A infected individuals seroconverting to a contemporary group A test virus was significantly higher than the proportion seroconverting to a contemporary group B virus ($p=0.0005$). The proportion of RSV B infected individuals seroconverting to a contemporary group B virus was higher than the proportion seroconverting to a contemporary A virus ($p=0.008$). The mean duration of maternal neutralising antibodies above a putative protective threshold was 2.8 months (95%CI 2.5–3.1 months). Primary neutralising antibodies declined to pre-infection levels within 3-4 months. The levels of some cytokines/chemokines correlated with viral load but not with RSV group.

Conclusion: The data suggest that population-level competition between RSV A and B is related to development of transient group-specific immunity following natural infection. Thus it might be argued that future RSV vaccines should include strains from both groups in order to maximise the indirect protective effect of a vaccination programme. No evidence was found for strain-specificity in cytokine/chemokine responses following RSV infection.

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Abbreviations

AEC	Amino ethylcarbazole
ADCC	Antibody Dependent Cell-mediated Cytotoxicity
ALRI	Acute Lower Respiratory Infection
CD	Cluster of Differentiation
CHD	Chronic Heart Disease
CI	Confidence Interval
CLD	Chronic Lung Disease
CPE	Cytopathic Effect
CTL	Cytotoxic T Lymphocyte
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Foetal Calf Serum
FI	Formalin-inactivated (RSV vaccine)
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GE	Gene End (sequence)
GS	Gene Start (sequence)
GST	Glutathione-S-transferase
HBD	Heparin Binding Domain
HIV	Human Immunodeficiency Virus

HLA	Human Leukocyte Antigen
HRP	Horseradish peroxidase
IFAT	Immunofluorescence Antibody Test
IFN	Interferon
IL	Interleukin
IP	Inpatient
KBC	Kilifi Birth Cohort
KDH	Kilifi District Hospital
MCHC	Maternal Child Health Clinic
MCP	Monocyte Chemoattractant Protein
MDC	Macrophage-Derived Chemokine
MDS	Multi Dimensional Scaling
MEM	Minimum Essential Media
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MRCA	Most Recent Common Ancestor
NK	Natural Killer (cells)
NOD	Nucleotide Oligomerization Domain
OPD	o-Phenylenediamine dihydrochloride
OR	Odds Ratio
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units

PRNA	Plaque Reduction Neutralisation Assay
PRNT	Plaque Reduction Neutralisation Titre
PRR	Pattern Recognition Receptor
RANTES	Regulated And Normal T cell Expressed and Secreted
RFLP	Restriction Fragment Length Polymorphism
RIG-1	Retinoic acid-Inducible Gene I
RLR	Retinoic acid-Inducible Gene I-like receptors
RNA	Ribonucleic Acid
RSV	Respiratory Syncytial Virus
RSVIG	RSV Immune Globulin
RT	Reverse Transcriptase
SD	Standard Deviation
SDS	Sodium dodecyl sulphate
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
TRIM-21	Tripartite Motif-containing 21
URTI	Upper Respiratory Tract Infection
WHO	World Health Organisation

Chapter 1 - General Introduction

The significance of RSV in the context of the global burden of respiratory illness due to bacterial and viral pathogens

Respiratory infections are a leading cause of the global infectious disease burden (Lozano *et al.*, 2012). Infections caused by such pathogens as *Mycobacterium tuberculosis*, *Streptococcus pneumoniae* and human influenza A virus have in the past exerted a heavy mortality and morbidity burden in different parts of the world, and for many continue to do so, particularly in low resource countries. For example, it is estimated that in 2000 approximately 14.5 million cases of severe pneumococcal disease occurred as a result of infection with *Streptococcus pneumoniae*, resulting in about 826,000 deaths worldwide among children who were less than 5 years of age (O'Brien *et al.*, 2009). *Haemophilus influenzae* type b which has been a leading cause of pneumonia in children is estimated to have caused about 8.13 million cases of serious illness worldwide in 2000, resulting in approximately 371,000 deaths among children who were less than five years of age (Watt *et al.*, 2009). It has been reported that in 2011, there were an estimated 8.7 million new cases of tuberculosis caused by *Mycobacterium tuberculosis* of which 13% were co-infections with HIV (World Health Organization, 2012). In the same year about 1.4 million deaths due to tuberculosis occurred, of which only about 430,000 were attributed to individuals with human immunodeficiency virus (HIV) (World Health Organization, 2012).

Respiratory infections caused by viruses have been associated with death and serious illness on a massive scale throughout the world. The influenza pandemic of 1918-1919 which was caused by an influenza A virus is thought to have infected about 500 million individuals worldwide with very high case fatality rates (Taubenberger and Morens, 2006). It is

estimated to have resulted in approximately 50 million deaths worldwide (Johnson and Mueller, 2002). More recently, the 2009 influenza A H1N1 pandemic is estimated to have resulted in 201,200 deaths from respiratory illness and 83,300 deaths as a result of cardiovascular complications (Dawood *et al.*, 2012).

Respiratory Syncytial Virus (RSV) is considered to be the most important viral cause of lower respiratory tract infection in infants and young children globally. It is estimated that in 2005 about 33.8 million new cases of acute lower respiratory infection attributable to RSV occurred among children who were less than 5 years of age (Nair *et al.*, 2010). Of these, at least 3.4 million episodes were of sufficient severity to necessitate hospitalisation. Between 66,000 and 199,000 children who were less than five years of age are estimated to have died in 2005 as a result of acute respiratory illness caused by RSV, with 99% of these deaths occurring in developing countries (Nair *et al.*, 2010). In Kenya for example, incidence rates for hospitalisation of RSV of 1,107 per 100,000 infants have been reported, with an estimated mortality rate of 2.2% among infants with severe RSV pneumonia (Nokes *et al.*, 2009). An analysis of the aetiology of severe pneumonia in children (figure 1.1) has shown that a large proportion of the severe pneumonia burden is attributable to RSV and other respiratory viruses relative to bacterial causes (Scott *et al.*, 2008). Viewed together, these estimates suggest that RSV is responsible for a significant proportion of the burden of respiratory illness caused by viral and bacterial pathogens, suggesting the need to design and conduct studies to understand both its clinical and epidemiological characteristics in order to develop effective control strategies.

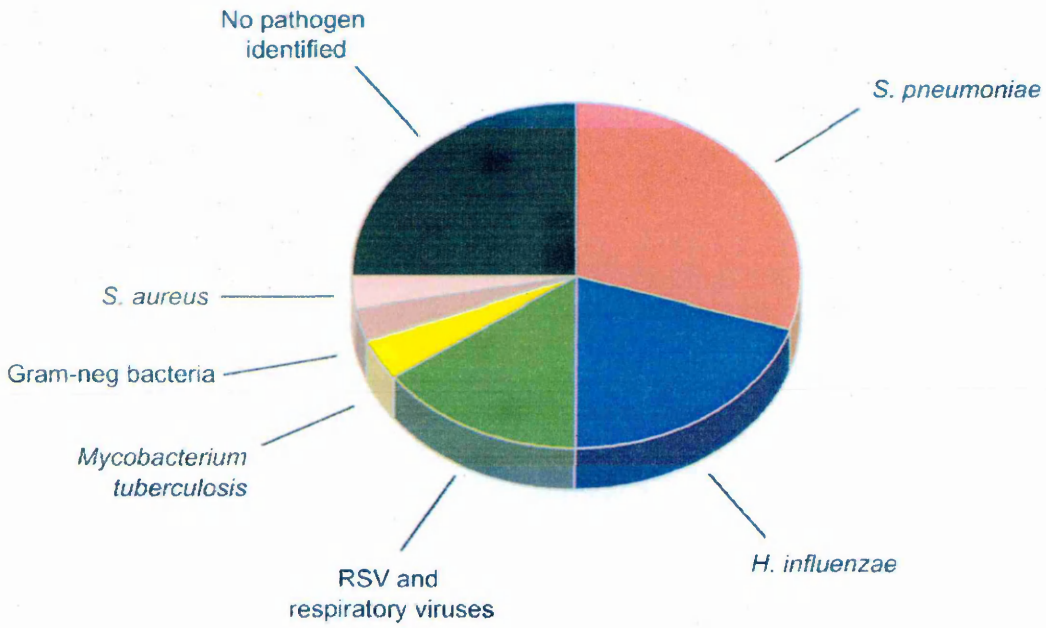

Figure 1.1 Pie chart showing the relative contribution of RSV and different bacterial pathogens in causing severe pneumonia in developing countries (Scott *et al.*, 2008).

Defining the question

RSV can be divided into two distinct groups A and B, which vary in terms of their sequence characteristics as well as in their reaction with certain monoclonal antibodies (Mufson *et al.*, 1985, Anderson *et al.*, 1985, Johnson *et al.*, 1987b). Within the group structure exist strains or genotypes that are variant in their sequence characteristics (Cane and Pringle, 1991). The transmission of RSV in most parts of the world is inherently cyclical and is characterised by regular outbreaks of acute respiratory infection at specific times of the year (Stensballe *et al.*, 2003). Studies on the strain structure of these epidemics have shown evidence of population level competition between the two antigenic groups A and B (Waris, 1991, Zlateva *et al.*, 2007) as well as between different genotypes (Cane, 2001). In some communities, this competition is manifested in alternating patterns of dominance of RSV A and B in successive epidemics (Waris, 1991, Zlateva *et al.*, 2007). This pattern of group and genotype replacement strongly implicates the involvement of an underlying selection mechanism.

A mechanism that potentially underlies the observed variations is immune selection. Following natural infection with one antigenic group, it is reasonable to assume that the resulting immune response would be more effective against the infecting group relative to the alternative group. If this host level response is extrapolated to the population level, it is likely that the transmission of one RSV group in a particular epidemic would lead to a build-up of population-level group-specific immunity, that would generally preclude the re-introduction of that group in the subsequent transmission season, but that would be permissible to the transmission of the alternative group.

RSV is known to repeatedly infect throughout life. The ability of the virus to re-infect may be related to antigenic variation, transient protective immunity or both. Recent studies have shown that repeat infections are often caused by viruses that are genetically different from those that caused the initial infections (Agoti *et al.*, 2012) although prior work had shown that re-infecting strains did not appear to be antigenically different from strains that had caused prior infection (Beem, 1967). The duration of protective immunity following primary RSV infection in infants has not been exhaustively investigated. There is general consensus that protective immunity correlates strongly with development of serum neutralising antibody (Glezen *et al.*, 1981a, Piedra *et al.*, 2003) suggesting the possibility that the duration of these antibodies correlates with the duration of protection. Previous studies have estimated that antibody responses to primary RSV decline to pre-infection levels within one year of infection (Welliver *et al.*, 1980, Kaul *et al.*, 1981). However, many of these studies were based on enzyme immunoassays that detected total antibodies and not neutralising antibodies which are likely to correlate more strongly with protection. The lack of data on the duration

of neutralising responses has hindered the ability to accurately predict the duration of protective immunity induced by natural primary infection.

The natural history of cytokine/chemokine responses following natural RSV infection is imperfectly understood. While animal models have provided in-depth data on the kinetics of different cytokines/chemokines in the course of infection (Sun *et al.*, 2011, Hussell *et al.*, 1996), there has been little validation of these results in infant disease. The most comprehensive data available in the human infant are based on samples obtained following the failed formalin-inactivated vaccine studies conducted in the 1960s. In this model severe disease was associated with development of an intense inflammatory infiltrate in the lungs of vaccinees who developed severe pneumonia following natural exposure (Openshaw and Tregoning, 2005). In addition to this, the role of RSV antigenic variation in driving differential cytokine/chemokine responses to RSV is not clearly understood. It is possible that variation in virus proteins that interact with elements of the host immune system could potentially result in variable cytokine/chemokine responses and ultimately variations in clinical disease.

The research presented in this thesis was designed to contribute to the understanding of the host immune response to RSV and its implications on both the transmission dynamics of the virus as well as in the modification of the host-virus interaction at the acute stage of illness.

Thesis research objectives

The key objective was to define the group and strain-specificity of the serum neutralising antibody response generated upon natural infection in infants. Two secondary objectives were to define the kinetics of the neutralising antibody response following natural infection and to

describe the natural history of different cytokine/chemokine cytokine/chemokine responses at the acute stage of RSV infection in infants, within the context of RSV strain diversity.

Specific objectives

1. Describe the development of the serum neutralising antibody response in the acute and convalescent phase of infection
2. Compare the partial G and F gene sequences of infecting RSV A and B viruses
3. Compare homologous and heterologous serum neutralising antibody responses to both RSV A and B
4. Evaluate the effect of the novel 60 nucleotide G gene duplication (BA genetic change) on the neutralising antibody response
5. Measure the average duration of neutralising antibodies following primary infection in infants
6. Evaluate the kinetics of different cytokine/chemokine responses over the course of natural RSV infection in infants and assess the effect of RSV strain variation on these responses

Approach

The research studies reported in this thesis were based on the measurement of neutralising antibody titres in both acute and convalescent phase sera obtained from infants with severe or very severe pneumonia admitted to a district hospital serving a rural coastal Kenyan community. Neutralising antibodies were also measured in serum obtained from a birth cohort that was recruited and followed up for up to 30 months of age, with active and passive surveillance of respiratory infection. Cytokine/chemokine responses were measured in nasal

samples collected at regular intervals prior to, during and after natural RSV infection in infants.

Declaration of author's role

The studies described in this thesis were designed by the author together with supervisors, Prof James Nokes, Prof Pat Cane and Prof Peter Openshaw. The author of this thesis was responsible for carrying out the laboratory assays and statistical data analysis. The publications that have arisen from this work are listed below:

1. Sande CJ, Mutunga MN, Medley GF, Cane PA, Nokes DJ. Group and genotype-specific neutralising antibody responses against respiratory syncytial virus (RSV) in infants and young children with severe pneumonia. J Infect Dis (Accepted)
2. White LJ, Sande CJ, Kinyanjui T, Cane PA, Waris M, Medley GN, Nokes DJ. Cross-immunity to human respiratory syncytial virus subgroups: *in vitro* measures inform molecular epidemiology (In preparation)
3. Sande CJ, Mutunga MN, Medley GF, Cane PA, Nokes DJ. The kinetics of the neutralising antibody response to respiratory syncytial virus infections in a birth cohort (In preparation)
4. Sande CJ, Mutunga MN, Medley GF, Cane PA, Nokes DJ. Neutralising antibody responses among infants with severe RSV pneumonia in Kilifi (In preparation)

Overview of the thesis

Chapter 2 of the thesis contains a review of the literature. The aim of this review is to provide a description of RSV in terms of its burden, transmission, molecular and antigenic

characteristics, and vaccine development. This chapter comprehensively reviews early published cross neutralisation studies that aimed to define the extent of antigenic heterogeneity between different strains of the virus. Chapter 3 provides a detailed description of the study design as well as the laboratory methods used in the different studies discussed in the thesis. Chapter 4 describes the development of a high throughput plaque reduction and neutralisation assay. Chapter 5 provides a description of the development of neutralising antibody responses following natural infection. In this chapter, age dynamics of the neutralising response are explored. The genetic characteristics of infecting strains are described in this chapter and finally, group homologous and heterologous neutralising antibody responses are compared. In Chapter 6, the kinetics of the RSV neutralising antibody response are described. Neutralising antibodies in a birth cohort recruited and prospectively followed up for approximately 30 months are described. The duration of the neutralising antibody response following natural infection is also described. Chapter 7 deals with genotype-specific neutralising antibody responses to RSV. In this chapter, neutralising responses to strains isolated between 40-50 years apart are assessed. Further, the effect of the recent 60 nucleotide duplication on the attachment protein gene of group B strains on the neutralising response is described. Chapter 8 describes the natural history of cytokine/chemokine responses following natural RSV infection in infants. In this chapter, the kinetics of different cytokines/chemokines over the entire course of natural RSV infection is described. The effect of strain variation on different cytokine/chemokine responses is also described in this chapter. Chapter 9 offers a summary and overall discussion of the main findings in this thesis. This chapter discusses limitations in the studies described and proposes potential avenues for future research.

Chapter 2 - Literature Review

Burden of disease of RSV

RSV is the most important viral cause of acute lower respiratory infection (ALRI) among infants globally. In 2005 at least 33.8 million cases of RSV associated ALRI occurred in the under 5 age group and of these, 3.4 million cases were associated with severe illness that necessitated hospital admission (Nair *et al.*, 2010). Between 66,000 and 199,000 deaths were estimated to have occurred in the same year as a result of RSV associated ALRI, with a vast majority of the deaths occurring in developing countries (Nair *et al.*, 2010). RSV mortality is associated with certain risk factors. A recent systematic review of co-morbidities associated with increased risk of death following RSV infection showed the mortality rate in children with congenital heart disease (CHD) was between 2 – 37%, while that of premature infants and those with chronic lung disease (CLD) was between 0 – 6.1% and 2 -37% respectively (Welliver *et al.*, 2010). These rates contrast sharply with the RSV associated mortality rate in otherwise healthy children which is reported to be less than 1% (Welliver *et al.*, 2010). Apart from its effect on the health of the patient, RSV imposes a heavy economic cost. In the United States for example, the average total cost of hospitalisation for term and pre-term infants infected with RSV exceeds the hospitalisation costs of infants admitted with other conditions by an average of $9,151 and $17,465 respectively (Palmer *et al.*, 2010). In view of the fact that the burden of severe RSV disease is highest among infants, these data suggest that the monetary cost associated with severe RSV infection among infants is substantial. The cost of RSV infection may also be measured in terms of days of work or school missed as a result of infection. Previous studies have shown that RSV infection in previously healthy adults is associated with periods of absence from work of up to one week

(O'Shea *et al.*, 2007, Hall *et al.*, 1978). As a result of the health and economic costs linked with infection, development of an RSV vaccine is considered to be a research priority.

Molecular structure of RSV

RSV belongs to the subfamily *Pneumovirinae* in the family *Paramyxoviridae*, order *Mononegavirales* (Pringle, 1997, Kingsbury *et al.*). RSV virions exhibit pleomorphic characteristics with virions budding from the plasma membranes of infected cells assuming a rounded or kidney shape of between 150 – 250 nm in diameter (Bachi and Howe, 1973). Alternatively budding virions may appear as filaments with a uniform diameter of 50 to 100 nm (Bachi and Howe, 1973). RSV is an RNA virus (Hamparian *et al.*, 1963) which contains 10 genes that code for 11 proteins (Dubovi, 1982, Huang *et al.*, 1985, Collins *et al.*, 1984). This is due to the fact that one of the 10 genes (M2) contains an internal overlapping open reading frame (ORF) that codes for an 11^{th} protein (M2-2) (Collins *et al.*, 1990a). The viral genome is of negative sense polarity (Lambert *et al.*, 1980, Huang and Wertz, 1982) and contains a functional polymerase within the viral nucleocapsid. Transcriptional mapping studies have demonstrated that the transcription of RSV occurs in a sequential manner in the order: NS1, NS2, N, P, M, SH, G, F, M2 and L (Dickens *et al.*, 1984). Figure 2.1 is an illustration of the molecular structure of RSV.

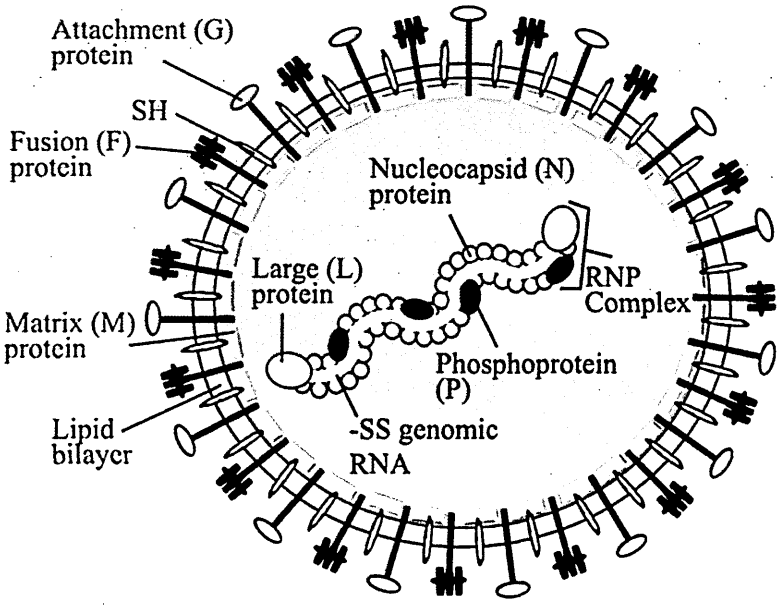

Figure 2.1 Schematic diagram of the structure of RSV (Empey *et al.*, 2010)

Sequential virus transcription by the viral polymerase is controlled by conserved gene start (GS) and gene end (GE) sequences at the beginning and end of every gene (Collins *et al.*, 1986). The RSV genome contains a single transcription promoter at the 3' region of the genome (Dickens *et al.*, 1984) which is used to initiate mRNA synthesis for all 10 genes. Once the polymerase binds to the promoter region, it initiates RNA synthesis and progresses until it encounters the GE region. This triggers the release of the nascent positive sense mRNA molecule that is specific for a particular viral protein (Cowton *et al.*, 2006) and that can be translated into protein by the host cell machinery. mRNA synthesis of the subsequent gene is initiated when the polymerase encounters the GS region of the gene and progressively synthesises a new mRNA strand until it encounters the GE region, where the process described above is repeated. This process progresses until the mRNA of the last gene on the RSV genome (L gene) is synthesised (reviewed by Cowton *et al.* (Cowton *et al.*, 2006)).

Viral proteins translated using the mRNA transcripts are then assembled into new, infectious virion particles along with a copy of the viral genome (Cowton *et al.*, 2006).

The epidemiology of RSV

The transmission of RSV is characteristically seasonal. In countries within the northern hemisphere with temperate climates, RSV typically causes epidemics of acute lower respiratory infection among infants and young children between the months of November and April. The temporal transmission cycles may occur at an annual (Hall *et al.*, 1990, Cane, 2001, Cane *et al.*, 1994, Hall *et al.*, 2009, Zlateva *et al.*, 2007) or biennial (Waris, 1991, Mlinaric-Galinovic *et al.*, 2008, Reyes *et al.*, 1997, Anestad, 1987) frequency depending on the geographical location. Countries with tropical and semitropical climate experience RSV at equally regular intervals but with different patterns of seasonality. In northern tropical countries, the increase of RSV transmission is associated with increase in rainfall and decrease in temperature (Cherian *et al.*, 1990, Weber *et al.*, 1998). In southern tropical countries, RSV epidemics are associated with decreases in both temperature and rainfall (Madhi *et al.*, 2006). In equatorial countries the association between climatic conditions and seasonal increases in transmission is less well defined. Some equatorial countries with perennial rainfall appear to experience RSV all year round (Reese and Marchette, 1991, Berman *et al.*, 1983, Tsai *et al.*, 2001), while others appear to experience seasonal epidemics at specific times of the year. The group replacement dynamics in Kilifi over a period of 9 years are depicted in Figure 2.2.

The onset of RSV epidemics in different geographical areas varies considerably. In general, RSV epidemics appear to occur in a south to north direction in the northern hemisphere and a north to south direction in the southern hemisphere (Stensballe *et al.*, 2003). In the United

States for example, over a 10 year period, epidemics on average began in the South on week 47 of the year followed by those in the Northeast at week 49, the West at week 52 and the Midwest at week 1 of the subsequent year. (Mullins *et al.*, 2003). Notably, the respective onset times of RSV seasons were found to vary widely year on year in the same geographical locations in the United States (Panozzo *et al.*, 2007).

Group replacement dynamics of RSV epidemics

RSV can be classified into two main groups A and B on the basis of both reaction with monoclonal antibodies and sequence data (Mufson *et al.*, 1985, Anderson *et al.*, 1985, Johnson, 1987). Each group can further be categorised into strains or genotypes which vary to a lesser extent than the 2 main groups, both at the nucleotide and amino acid sequence level. Table 2.1 shows the extent of nucleotide and amino acid identity in some virus genes.

Protein	Nucleotide identity (%)	Amino acid identity (%)	Reference
G	67	53	(Johnson *et al.*, 1987b)
F	79	91	(Johnson and Collins, 1988)
SH	78	76	(Collins *et al.*, 1990b)
NS1	78	87	(Johnson and Collins, 1989)
M2	78	92	(Collins *et al.*, 1990a)
NS2	78	92	(Johnson and Collins, 1989)
N	86	96	(Johnson and Collins, 1989)

Table 2.1 Nucleotide and amino acid identity estimates between RSV A and B strains

RSV epidemics exhibit extensive genotype variability. Generally RSV A strains appear to circulate at a higher frequency than RSV B strains throughout the world (Hall *et al.*, 1990, Reiche and Schweiger, 2009, Zhang *et al.*, 2010, Zlateva *et al.*, 2007). Studies of the group replacement dynamics in successive RSV epidemics have shown that the two RSV groups cyclically alternate in a generally predictable manner. For example, data collected in England and Wales over an 11 year period showed a cyclic triennial pattern in RSV A and B transmission (Cane, 2001). A similar pattern of population level competition between RSV A and B in successive epidemics has also been reported in Belgium (Zlateva *et al.*, 2007). The pattern of cyclic alternation in Finland is markedly different from that in England and Wales and Belgium, where the dominant group alternates every two years (Waris, 1991). In Uruguay between two and three successive epidemics of RSV A are followed by one RSV B epidemic (Arbiza *et al.*, 2005) while in France and Boston in the United States, less regular patterns of group replacement have been reported in which successive seasons of co-dominance are followed by dominance by either RSV A or B (Hendry *et al.*, 1989, Freymuth *et al.*, 1991). The mechanisms though which certain epidemics display co-dominance in transmission between strains from the two groups are not understood. While determinants such as relative reproductive rates, degree of cross-protection and rate of waning immunity will be important (White *et al.*, 1998) stochastic effects will undoubtedly play a role. It might be speculated that co-dominance could by chance arise due to the proportion of the population that is exposed to infection in any given epidemic. For example if only half the population is exposed to infection by one group during an epidemic while the other half remains un-infected, it may be that in the subsequent epidemic, the previously exposed group would be at an elevated risk of infection with the alternative group, while individuals who were not infected in the previous epidemic could be equally at risk of infection by both RSV A and B.

Studies of the genotype diversity of RSV epidemics in different parts of the globe have yielded largely similar results. Multiple genotypes appear to constitute most epidemics, with the predominant genotype being successively replaced from one year to the next. For example a study in Birmingham in the United Kingdom found that different genotypes of both RSV A and B were predominant in each of 5 successive epidemics between 1988 and 1993 and that the predominant genotypes appeared to decline over time and were replaced by alternative genotypes in epidemics that followed (Cane *et al.*, 1994). These data are graphically shown in figure 2.3. A Korean study that looked at genotype prevalence over 9 consecutive epidemics found that multiple genotypes were in circulation and the predominant genotype in each epidemic was replaced in subsequent epidemics. Phylogenetic analysis of sequences in this study as well as others derived from other parts of the world, showed that similar strains were simultaneously circulating in other parts of the world (Choi and Lee, 2000). These observations were supported by the findings of Cane *et al.* who also reported on the homogeneity of simultaneously circulating genotypes in different parts of the world (Cane *et al.*, 1992). Seki *et al.* looked at genotype diversity over 15 successive epidemics in Japan and found that that the dominant genotypes were similarly replaced in subsequent epidemics (Seki *et al.*, 2001). Similar findings were reported in two separate studies of hospitalised and ambulatory children in Rochester, New York (Hall *et al.*, 1990, Peret *et al.*, 1998). These observations have led to suggestions of the existence of population level competition between the two RSV groups as well as competition between different genotypes and that a build up of group/genotype-specific immunity at population level underlies the observed group/genotype replacement patterns in consecutive epidemics (Cane, 2007).

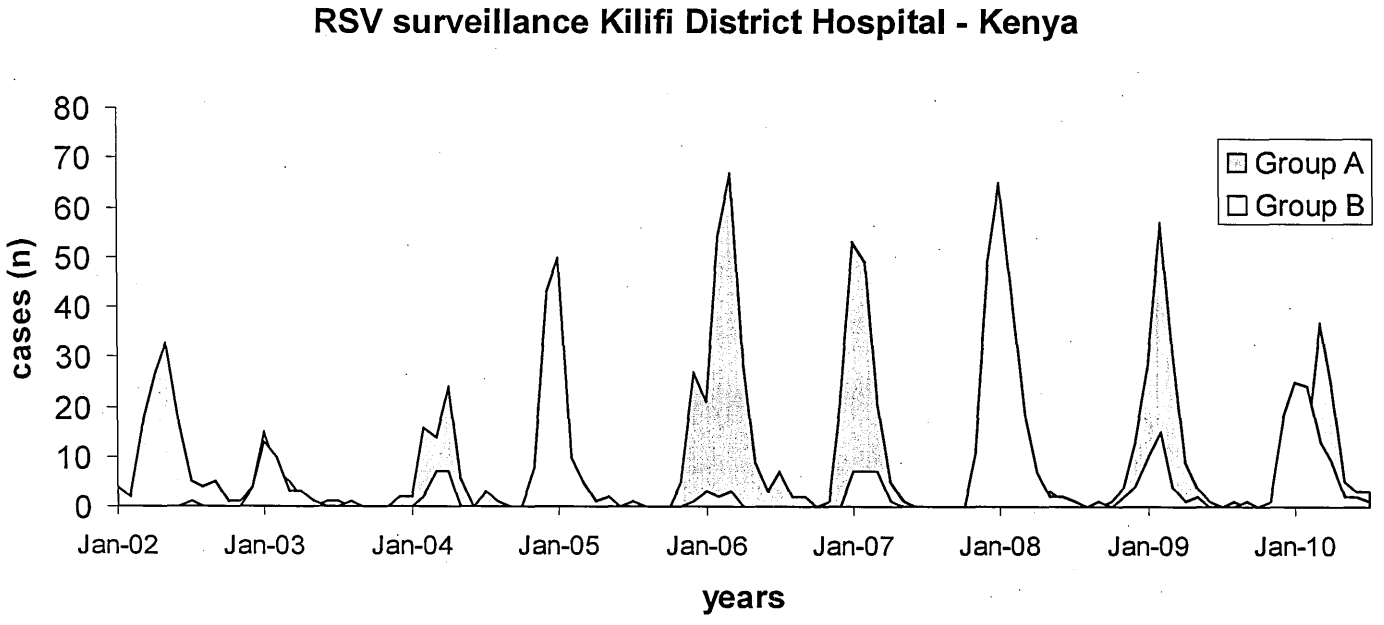

Figure 2.2 Occurrence of RSV epidemics over a nine year period of surveillance. Of the nine epidemics that occurred with this period, 5 were predominantly RSV A epidemics (i.e. the epidemics of 2001/2002, 2003/2004, 2005/2006, 2006/2007 and 2008/2009), 2 RSV B (i.e. the epidemics of 2004/2005 and 2007/2008) , while 2 appeared to have been co-dominated by both RSV A and B (i.e. epidemics of 2002/2003 and 2009/2010) (Nokes *et al.*, In Preparation).

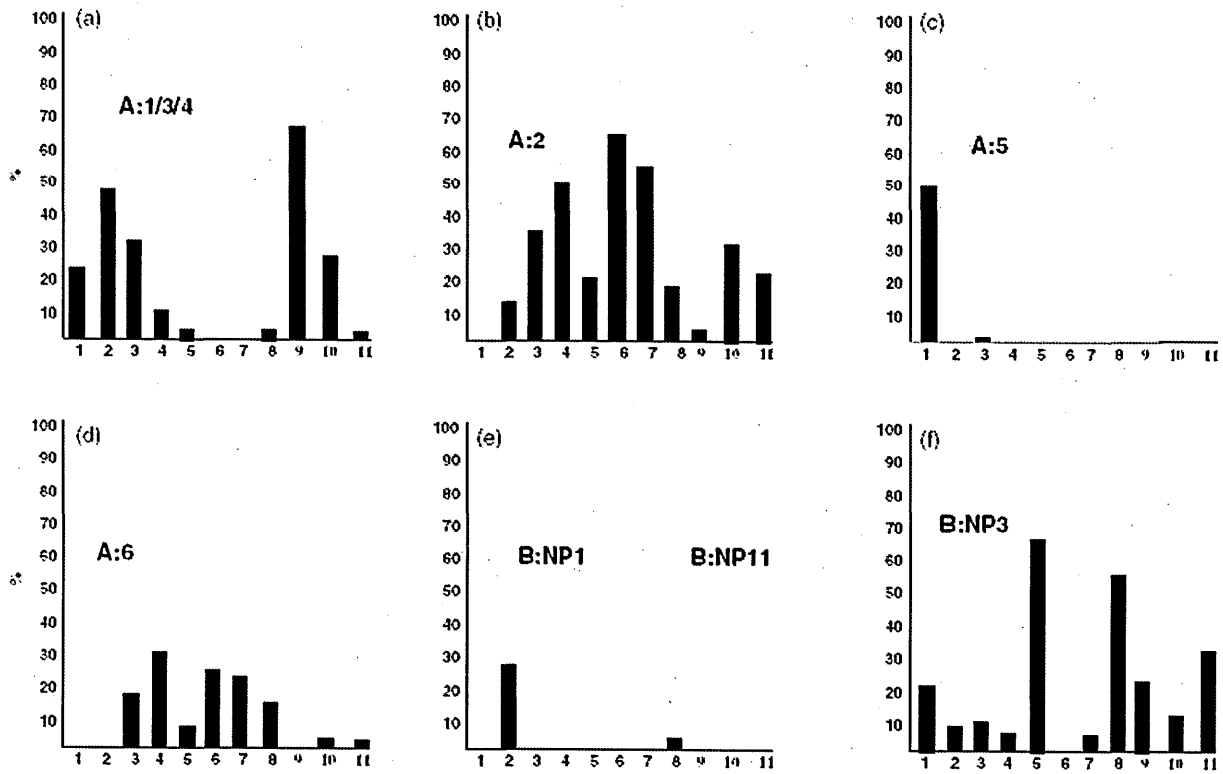

Figure 2.3 The pattern of genotype replacement over a period of 11 epidemics in Birmingham, United Kingdom (Cane *et al.*, 1994).

Role of G and F proteins in virus binding and membrane fusion

Many paramyxoviruses gain entry into the cell through fusion of the virus and cell membranes (Chang and Dutch, 2012). In the case of RSV, prior to the fusion process, the virus through the G protein has been shown to attach to nucleolin on the surface of susceptible cells (Tayyari *et al.*, 2011). This conclusion is based on the fact the ability of the virus to infect cells *in vitro* is significantly reduced in the presence of anti-nucleolin antibodies, soluble nucleolin and RNA interference that is targeted at reduced intracellular nucleolin expression (Tayyari *et al.*, 2011). In addition to nucleolin, entry is likely to be facilitated by binding of the virus to heparin which is a soluble proteoglycan on the cell surface (Krusat and Streckert, 1997). The binding of RSV to heparin and other proteoglycans appears to be through recognition of the glycosaminoglycan (GAG) moieties of proteoglycans (Hallak *et al.*, 2000, Martinez and Melero, 2000). Heparin sulphate has been suggested to be the main GAG involved with this entry mechanism (Hallak *et al.*, 2000, Martinez and Melero, 2000). There is evidence that suggests that the interaction of RSV with GAGs on the host cell surface occurs through the G protein (Techaarpornkul *et al.*, 2002). The region of the G protein consisting of amino acid residues 184-198 for RSV A and 183-197 for RSV B has been identified as the heparin binding domains (HBD) for human RSV (Feldman *et al.*, 1999).

The role of the G protein in the attachment process has been investigated using recombinant viruses in which the G protein has been deleted. Recombinant viruses lacking the G protein have been shown to replicate efficiently *in vitro* in a cell-line dependent manner but poorly in mice which are intranasally inoculated with the virus (Teng *et al.*, 2001). This suggests that the G protein may be dispensable in the attachment and entry process in some cells but is required for efficient replication *in vivo*. The role of the central conserved region in the

attachment process has been investigated using recombinant viruses containing a deletion of this region. Surprisingly in these studies it was found that deletion of the central conserved region did not reduce the efficiency of attachment to susceptible cell lines (Teng and Collins, 2002) suggesting that despite being conserved this region does not play a significant role in the interaction between RSV and it's cell surface receptors. The role of the conservation of the central part of the protein in the attachment process remains to be explained.

Upon attachment of the virus to the host cells, fusion of the virus and cell membranes takes place following activation of the F protein. The process of activation of the F protein is thought to be dependent on close proximity of the protein to the host cell membrane since this process involves exposure of the fusion peptide (Melero, 2007). This proximity requirement is crucial for successful infectivity since without it the hydrophobic fusion peptide is instead inserted into the virus membrane thus abrogating its infectivity (Lamb and Jardetzky, 2007). Upon binding to the host cell, the fusion peptide is exposed and inserted into the cell membrane and subsequently folds, bringing the cell and virus membrane into close proximity. This is followed by mixing of the two membranes and formation of the fusion pore, that connects the interior of the of the virus to the host cell's cytoplasm (Melero, 2007).

The role of innate and cell mediated immune responses in RSV infection

Activation of the host innate immune system by viruses typically occurs through the interaction of virus with pattern-recognition receptors (PRRs) on the host's innate immune cells such as dendritic cells and macrophages (Medzhitov, 2007). PRRs bind to viral molecules such as double-stranded RNA (dsRNA) (Yoneyama *et al.*, 2004), single-stranded RNA (ssRNA) (Pichlmair *et al.*, 2006), RNA with 5'-triphosphate ends (Hornung *et al.*, 2006) and proteins of viral origin (Thompson *et al.*, 2011). Among the PRRs known to

interact with viral components are Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Takeuchi and Akira, 2009). RLRs and TLRs are important for the stimulation of type I IFNs (Kato *et al.*, 2005) which lead to elimination of viral components from infected cells. Activation of these receptors also leads to production of pro-inflammatory cytokines and chemokines which provoke inflammation and recruit innate and acquired immune cells to the site of infection (Kumar *et al.*, 2006). In response to the inflammatory response mounted by the host, RSV has evolved mechanisms to antagonise these responses. The NS1 and NS2 proteins have been shown to have an inhibitory role in the induction of interferon alpha, beta and gamma responses by the host (Spann *et al.*, 2004).

T cells have a key role in the clearance of viral infection and exhibit a number of effector functions that are crucial in viral clearance. For example CD8+ effector T cells can provide complete protection against influenza infection in the absence of B cell specific responses (Graham and Braciale, 1997). The importance of T cell responses in protection is underscored by murine studies that have shown that cytotoxic T lymphocytes (CTLs) play a key role in the clearance of RSV infection (Cannon *et al.*, 1988). Individuals with defects in T cells responses do not effectively control RSV infection, further suggesting a clear role for T cell specific responses in viral clearance (Hall *et al.*, 1986).

Immunity to RSV: targets of protective antibody responses

The surface of the infectious virion contains 3 virus encoded proteins; F, G and SH that are exposed to the host immune system. Studies with infant and monoclonal antibodies have confirmed that these surface proteins, are the targets of antibody responses (Cote *et al.*, 1981, Akerlind-Stopner *et al.*, 1993) suggesting that they might be targets of protective immunity. Two of these proteins, F and G are thought to be targets of the neutralising antibody response.

The mechanisms through which virus neutralisation is thought to occur as well as description of the antigenic characteristics of the F and G proteins are discussed below.

Mechanisms of virus neutralisation

Virus neutralisation can broadly be defined as the abrogation of virus infectivity by antibody mediated mechanisms. The mechanisms through which antibodies neutralise virus can be classified on the basis of the event within the replication cycle of the virus that is blocked by such antibodies. A number of these mechanisms are discussed below.

Attachment of the virus onto host cells is crucial for successful infection. Antibodies that block this step of the infection process effectively curtail the ability of the virus to infect the cell (Burton *et al.*, 2000). Abrogation of infectivity can also be mediated by antibodies that block both conformational changes and un-coating. This mechanism of neutralisation has been well demonstrated in the case of poliovirus (Wetz, 1993). Some neutralising antibodies act by preventing intermediate steps in the virus life cycle. For example, some poliovirus neutralising antibodies have been found to prevent the formation of the 135S particle (Vrijsen *et al.*, 1993), an essential intermediate in the entry of poliovirus into the cell (Fricks and Hogle, 1990). Neutralisation of poliovirus has also been associated with aggregation of infectious particles by antibodies, with residual infectivity being associated with un-aggregated virions (Thomas *et al.*, 1986). Neutralising antibodies have recently been found to mediate inactivation of virus through cytosolic degradation. A recent study has shown that antibody coated adenoviruses are transported into the cytosol where they bind to an intracellular cytosolic protein called tripartite motif-containing 21 (TRIM21). TRIM21 has been demonstrated to bind with high affinity to an invariant region of antibody molecules (James *et al.*, 2007). Virus-antibody complexes within the cytosol bind to TRIM21, which

targets these complexes to the proteasome for degradation (Mallery *et al.*, 2010). Virus infectivity can also be effectively curtailed through antibody dependent cell-mediated cytotoxocity (ADCC). ADCC is a mechanism through which immune effector cells target infected cells for lysis through the recognition of antibody on the surface of infected cells. ADCC has been shown to be mediated by natural killer (NK) cells, macrophages, neutrophils and eosinophils. In the case of RSV, specific ADCC responses have been observed as early as 3 days following infection and peak at between 2 to 4 weeks after the onset of illness (Kaul *et al.*, 1982). Direct cell killing of RSV infected cells has also been reported to be mediated through complement. Lysis of RSV infected cells has been shown to involve both the classical and alternative complement activation pathways (Edwards *et al.*, 1986). Kaul *et al.* showed that complement mediated killing of RSV infected cells is enhanced in the presence of neutrophils. This complement dependent cytotoxicity is not as pronounced in the presence of lymphocytes or monocytes (Kaul *et al.*, 1984). Antibody mediated complement activation has also been linked to virus clearance. Antibodies that are defective in complement activation do not reduce lung titres in RSV challenged mice relative to antibodies that are capable of fixing complement, strongly suggesting that antibody activation of complement may play a key role in viral clearance upon infection (Mekseepralard *et al.*, 2006).

There is evidence that suggests RSV may escape the host neutralising antibody response through generation of escape mutants. Previous studies have shown that neutralising monoclonal antibodies directed at both the G and F protein can be used to select for escape mutants that are no longer recognised by the selecting antibodies (Garcia-Barreno *et al.*, 1990, Zhu *et al.*, 2011). Analysis of the viruses shed by children who are under palivizumab immunoprophylaxis have shown the development of escape mutations against this

immunoprophylactic antibody (Papenburg *et al.*, 2012). It is possible that the lack of proofreading ability by the virus' RNA polymerase might lead to the generation of a large diversity of genetically distinct viruses during replication. Through a process of natural selection, only the variants that are capable of surviving the prevailing antibody pressure would then be selected for replication and ultimately transmission as escape mutants.

The G Protein

The G protein is the virus attachment protein (Levine *et al.*, 1987), which mediates attachment of the virus to a cell surface receptor recently identified to be nucleolin (Tayyari *et al.*, 2011). The G protein is synthesised as a 33 kD precursor which is extensively modified by the addition of N and O linked carbohydrates (Gruber and Levine, 1985a). These carbohydrates contribute about 57 kD to the molecular weight of the mature protein which is estimated to be about 90 kD (Gruber and Levine, 1985a). The protein is approximately 300 amino acids long although this may vary depending on the strain. Two forms of the protein are synthesised in the course of infection; a membrane anchored form and a soluble form (Hendricks *et al.*, 1987). The soluble forms are 6-9 kD smaller than the membrane anchored forms (Hendricks *et al.*, 1988) and are translated through an alternative in-frame start codon (Roberts *et al.*, 1994). The soluble form of the molecule is thought to act as an antigenic decoy, by binding to host antibody and thus helping the virus to evade antibody-mediated restriction both *in vivo* and *in vitro* (Bukreyev *et al.*, 2008). The G protein ectodomain can be divided into three distinct segments; a central conserved segment sandwiched between two hypervariable domains on the N and C terminals of the protein (Cane *et al.*, 1991). The central conserved region contains a 13 amino acid motif (residues 164 – 176) as well as 4 cysteine residues (173, 176, 182 and 186) that are perfectly conserved in all human RSV isolates (Melero *et al.*, 1997, Cane *et al.*, 1991). The high degree of sequence conservation in

this region is strongly suggestive of functional constraint and the region may therefore constitute an effective target for protective antibody responses.

The antigenic structure of the G protein

Identification of antibody epitopes on the primary structure of the G protein has been achieved mainly through reactivity with murine monoclonal antibodies. Three distinct epitope groups on the G protein have been identified using this approach: conserved, group-specific and strain-specific epitopes (Martinez *et al.*, 1997). Antibodies to conserved epitopes bind to all RSV strains, while group-specific antibodies bind to all strains from one of the two antigenic groups. Strain-specific antibodies bind to only certain strains of an antigenic group.

Escape mutants selected using monoclonal antibodies to conserved epitopes (i.e. antibodies that bind to all strains of RSV) contain amino acid changes in the central part of the G molecule (Martinez *et al.*, 1997, Rueda *et al.*, 1994, Walsh *et al.*, 1998) including the loss of one of the four conserved cysteines (Rueda *et al.*, 1994), suggesting that conserved epitopes are located on the central part of the molecule. On the other hand, escape mutants selected by antibodies to strain-specific epitopes contain amino acid substitutions (Martinez *et al.*, 1997), premature stop codons (Rueda *et al.*, 1991) or frame shift mutations (Garcia-Barreno *et al.*, 1990) in the carboxy terminal region of the G protein. Antibodies to the G protein are thought to recognise linear rather than conformational epitopes. This assertion is based on the fact that no murine monoclonal antibodies have so far been found to select for non synonymous substitutions at sites that are distant from their putative epitopes (Melero *et al.*, 1997). Antibodies to the G protein have also been shown to bind to overlapping stretches of synthetic peptides (Cane, 1997) covering different regions of the G protein, further

suggesting the lack of a conformational requirement for antibody binding. The genetic and antigenic structure of the G protein is graphically depicted in figure 2.4.

Sequence conservation in the central part of the G molecule suggests, as previously discussed, that it may be functionally constrained and possibly the target of cross-reactive antibody responses. Up to 40% of adults with natural RSV infection (Murata *et al.*, 2010) generate antibody responses to the central part of the G molecule. This region is the target of a partially neutralising cross-reactive monoclonal antibody (Walsh *et al.*, 1989) providing grounds for inferring the importance of responses to this region to neutralising antibody protection.

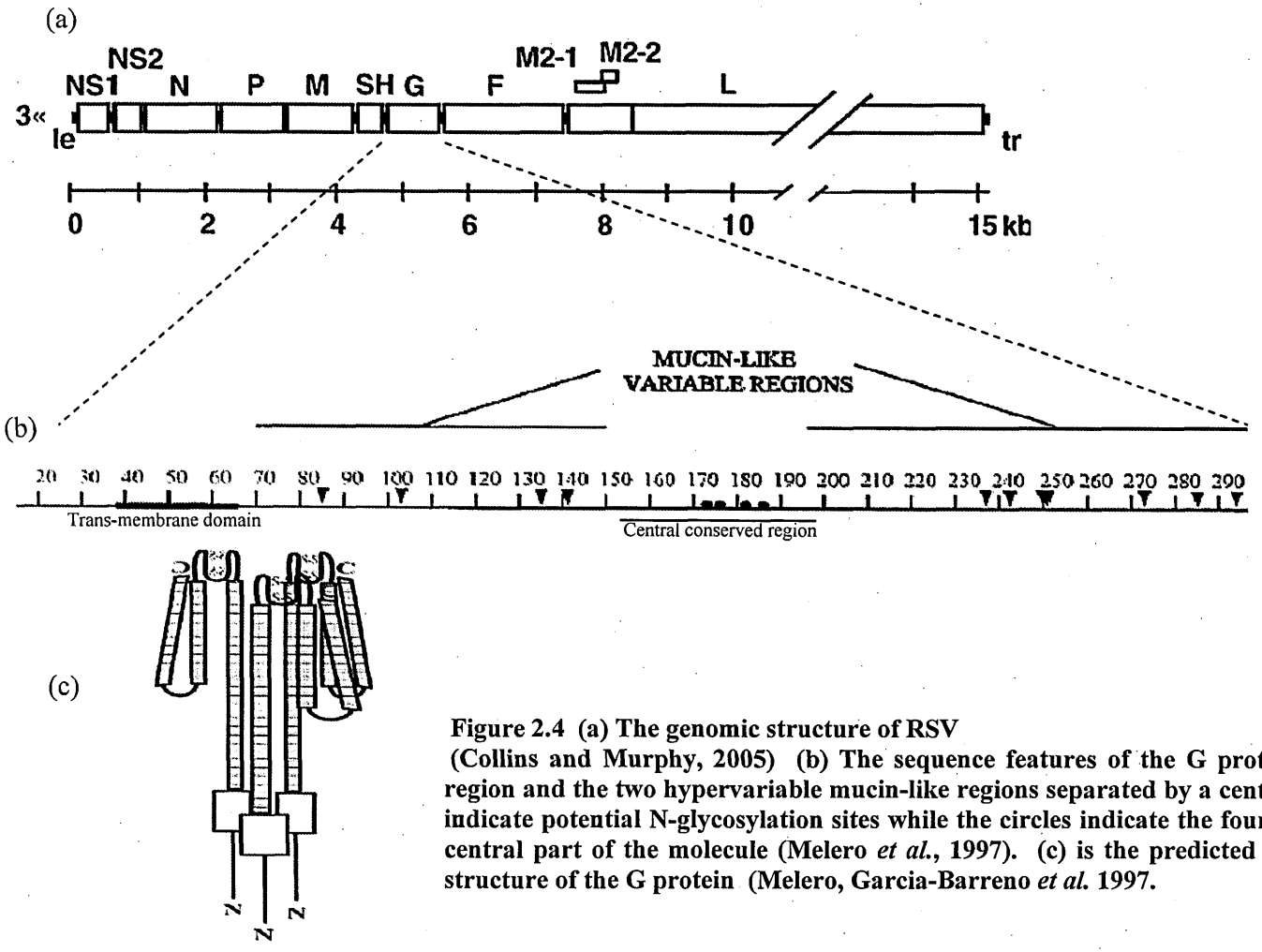

Figure 2.4 (a) The genomic structure of RSV (Collins and Murphy, 2005) (b) The sequence features of the G protein including the trans-membrane region and the two hypervariable mucin-like regions separated by a central conserved region. The triangles indicate potential N-glycosylation sites while the circles indicate the four conserved cysteine residues in the central part of the molecule (Melero *et al.*, 1997). (c) is the predicted structure of the three dimensional structure of the G protein (Melero, Garcia-Barreno *et al.* 1997.

Human antibody responses to the G protein

Due to its extensive variability, antibody responses to the G protein are thought to be genotype-specific (Johnson *et al.*, 1987a, Anderson *et al.*, 1985, Walsh *et al.*, 1987, Hendry *et al.*, 1988, Yamazaki *et al.*, 1994, Cane, 1997). Enzyme linked immunosorbent assays carried out using acute and convalescent phase sera from infants with acute RSV infection show that the group homologous infant response to purified F protein is cross-reactive but the infant response to the G protein is strongly group-specific (Hendry *et al.*, 1988). Due to the extensive glycosylation on the G protein, several studies have looked at the role of carbohydrate moieties on the infant response to the G protein. Wagner *et al.* found that the infant IgG response to both the G and F protein were primarily of the IgG1 and IgG3 subclass (Wagner *et al.*, 1986) and that the infant IgG2 response to the G and F proteins was generally poor (Wagner *et al.*, 1986). The lack of an infant IgG2 subclass response to these glycoproteins was surprising since the natural IgG2 response is typically directed at carbohydrate antigens while IgG1 and IgG3 responses are typically directed at protein antigens (Barrett and Ayoub, 1986). In contrast, the adult anti-G response is characterised by high production of both IgG1 and IgG2 (Wagner *et al.*, 1987b). The results of these studies suggest that the protein moieties on the G glycoprotein may have an immunodominant role over sugar moieties in the infant response and that recognition of the sugar moieties of the G glycoprotein appears to progressively increase with age. Studies of human serum responses to pneumococcal polysaccharide vaccines have confirmed this age structured response to carbohydrate antigens; the IgG1:IgG2 ratio decreases significantly with age in response to vaccination with both the polysaccharide and protein-polysaccharide conjugate *Streptococcus pneumoniae* vaccines (Lottenbach *et al.*, 1999).

A number of studies have looked at the antigenic specificity of infant serological response to the G protein following natural infection. Norrby *et al.* investigated the serum responses of infants with natural infections against a series of 23 overlapping peptides spanning the entire length of the G protein ectodomain (Norrby *et al.*, 1987). They reported that only three of these peptides reacted with human sera and none of these was located in the variable region of the protein (Norrby *et al.*, 1987). A possible reason for the failure to recognise the variable regions of the G protein could have been the fact that the infant responses were to wild-type strains that could have been antigenically different from the A2 strain that was used as a template for peptide synthesis. To address this possibility, Cane *et al.* looked at infant serological responses to Glutathione-S-Transferase (GST) fusion proteins representing 84-85 amino acids on the variable carboxy terminal third of 6 contemporary isolates of RSV A (Cane *et al.*, 1996). They reported that over half of the infants recognised these proteins, suggesting that the lack of recognition of this region in the work reported by Norrby *et al.* could be explained by the lack of antigenic similarity between the test and infecting antigens, possibly as a result of antigenic drift within this region over time. Interestingly, the pattern of recognition of this variable region of the protein was closely related to the infecting genotype (Cane *et al.*, 1996) with infants generating greater responses to sequences that more closely resembled the infecting strains (Cane *et al.*, 1996). These data are shown in figure 2.5. Further work on the infant serological response against overlapping peptides spanning the terminal 84-85 amino acids of the carboxy terminal end of the protein, showed that single amino acid mismatches could abrogate antibody binding (Cane, 1997), providing further evidence of the genotype-specificity of the antibody response to the variable region of the G protein. More recent evidence shows that the antibody repertoire to this region appears to generally broaden with repeated exposure (Scott *et al.*, 2007).

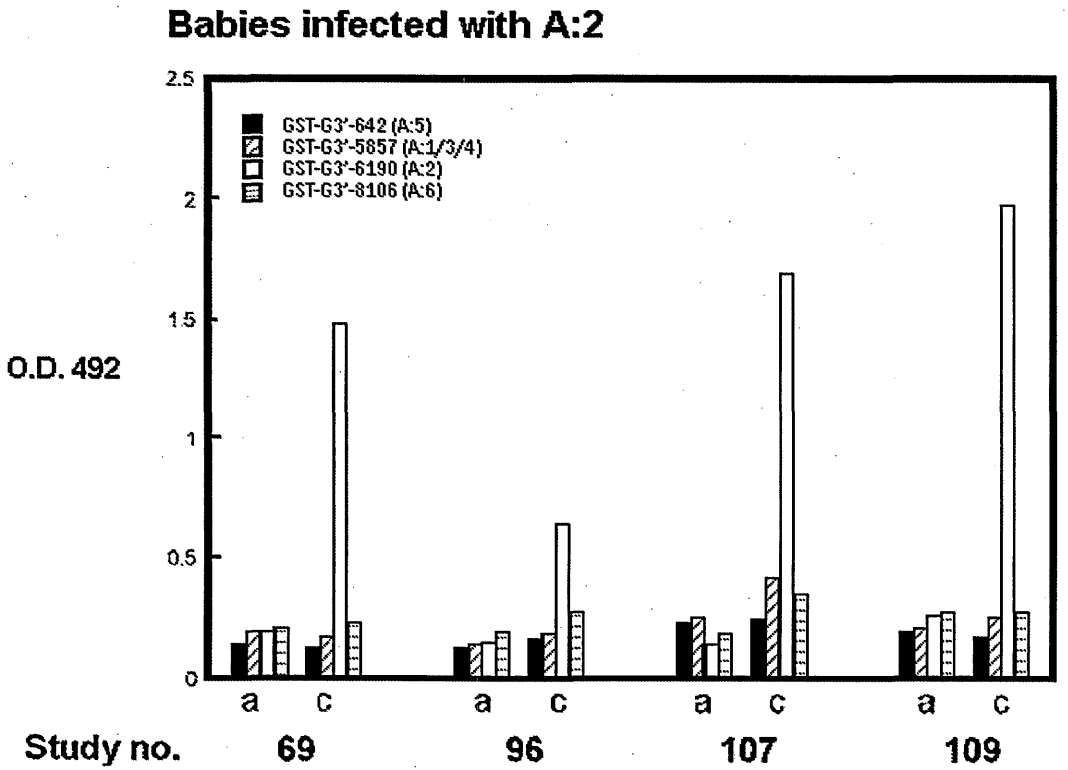

Figure. 2.5 Infant responses to GST fusion proteins representing the carboxy terminal third of the G protein. The acute (a) and convalescent (c) phase sera of each child was tested against a panel of the GST fusion proteins indicated on the legend on the top left of the graph. In this example, babies infected with the A:2 strain of the virus clearly make significantly greater responses to the A:2 GST fusion protein, relative to the others (Cane *et al.*, 1996).

The F protein

The F protein is the viral fusion protein (Walsh and Hruska, 1983). It mediates cell to cell fusion of virus infected cells leading to syncytia formation, a characteristic of RSV infected cells (Walsh and Hruska, 1983). The protein is synthesised as an inactive 69kD precursor (F_0) (Gruber and Levine, 1985a, Gruber and Levine, 1983) which is co-translationally modified in the endoplasmic reticulum by the addition of N-linked carbohydrates (Gruber and Levine, 1985a, Collins and Mottet, 1991). It is then cleaved into two subunits F1 (49 kD) and F2 (20 kD) which remain linked by a disulphide bond (Gruber and Levine, 1985b) and are subsequently transported to the cell surface (Collins and Mottet, 1991).

Antigenic structure of the F protein

Mapping of antibody binding sites on the primary structure of the fusion protein has been done using two approaches: generation and sequencing of monoclonal antibody escape mutants and use of synthetic peptides to examine regions of binding by monoclonal antibodies along the length of the protein. Arbiza *et al.* used these approaches to define two antigenic sites along the F protein that are targets of antibody responses. The first site contained a number of overlapping epitopes on the amino terminal third of the F1 subunit while the second was located on the carboxy terminal end of the F1 subunit's cysteine rich region (Arbiza *et al.*, 1992). Sequence comparison between the wild-type RSV Long strain and the monoclonal antibody escape mutants showed that a series of non-synonymous substitutions between amino acid position 190 and 272 were key to antibody escape in the first region while non-synonymous substitutions between amino acid 429 and 447 were responsible for antibody escape in the second region (Arbiza *et al.*, 1992, Lopez *et al.*, 1998).

There is evidence that suggests that antibodies to the F protein recognise conformational rather than linear epitopes. The conformational nature of epitopes on the F protein can be demonstrated by an analysis of the positions of amino acid substitution required for abrogation of antibody binding. Arbiza *et al.* reported the generation of a monoclonal antibody escape mutant in which a non-synonymous amino acid substitution that was necessary for antibody escape, occurred 57 amino acids upstream of the putative monoclonal antibody binding site (Arbiza *et al.*, 1992, Lopez *et al.*, 1993). Further evidence of the conformational requirement for F protein antibodies has been obtained from studies using synthetic peptides where it has been found that some F-specific antibodies only bind to long peptides and fail to bind to shorter peptides spanning the length on the longer one (Lopez *et al.*, 1993), suggesting that these antibodies can only bind to a peptide that is long enough to fold upon itself. More evidence of the need for higher order structures for antibody binding to the F protein has been shown in experiments in which fragments of the F protein are pre-treated with Sodium Dodecyl Sulphate (SDS) prior to reaction with antibodies. The results of these experiments have shown that pre-treatment with SDS – which linearizes proteins by conferring a net negative charge – leads to loss of antibody binding (Lopez *et al.*, 1993). Figure 2.6 is a summary of the antigenic structure of the F protein.

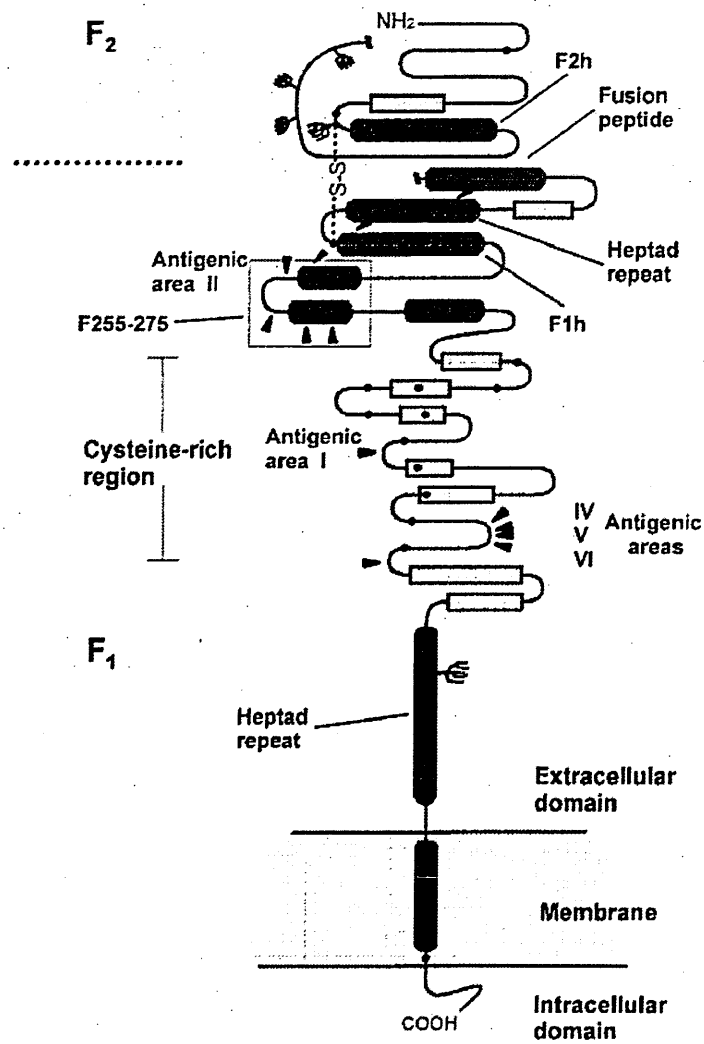

Figure 2.6 The predicted antigenic structure of the F protein (Lopez *et al.*, 1998).

Antibody responses to the F protein

Neutralisation studies with monoclonal antibodies suggest that the F protein is the main target of cross-reactive neutralising antibodies. These studies show that only F-specific monoclonal antibodies mediate complete neutralisation of RSV *in vitro* while antibodies to the G protein mediate incomplete or strain-specific neutralisation (Anderson *et al.*, 1988, Garcia-Barreno *et al.*, 1989). Passive transfer of F-specific monoclonal antibodies in mice and cotton rats infected with RSV significantly reduces the titre of virus in the lungs of these animals relative to those of controls (Taylor *et al.*, 1984, Walsh *et al.*, 1984) suggesting that F-specific antibodies have a protective effect *in vivo*. Immunisation of cotton rats with recombinant vaccinia viruses expressing F protein has been shown to lead to development of neutralising antibody titres that are up to 6-fold greater than the response to recombinant vaccinia virus expressing the G protein (Olmsted *et al.*, 1986), further supporting the notion that the F protein is the major target of neutralising antibody responses.

Potential mechanisms of protection by F and G specific antibodies

The availability of the immunoprophylactic monoclonal antibody palivizumab has provided the opportunity to speculate on the potential mechanisms of protection by F specific antibodies. While administration of palivizumab has been shown to significantly reduce the risk of developing severe disease (The IMpact-RSV Study Group, 1998), infants under palivizumab immunoprophylaxis do not appear to have a reduced risk of infection since these infants often become infected despite administration of the antibody (Papenburg *et al.*, 2012, Zhu *et al.*, 2011). This suggests that F specific antibodies are important in the prevention of severe disease but not infection. The role of G specific antibodies in mediating protection against either infection or disease in humans remains to be explained.

Antigenic characteristics of RSV

Early cross neutralisation studies

The question of whether the human neutralising antibody response to RSV is group or genotype-specific has been the subject of a number of important studies, but which has to date not been conclusively answered. In an attempt to define the group-specificity of the neutralising response, Coates *et al.* conducted studies in both human subjects and animal models (Coates *et al.*, 1963). Separate groups of ferrets were infected with representative strains from the two groups i.e. the Long strain (an RSV A strain isolated in 1956 in Baltimore, (Coates *et al.*, 1963)) and the CH 18537 strain (an RSV B isolated in 1962 Washington D.C. (Coates *et al.*, 1963)) and the resulting post-infection hyperimmune sera were then tested for cross neutralising ability. They found that the ferret post-infection response was group-specific with strong evidence that the animals generated a significantly greater homologous to heterologous neutralising antibody response (Coates *et al.*, 1963). The second arm of the study looked for a similar response in infant convalescent sera following natural infection. Infants with serological evidence of RSV infection who were hospitalised with a diagnosis of bronchiolitis or pneumonia in the 1956-57 RSV season in Baltimore as well as those hospitalised in the 1960 and 1962 RSV seasons in Washington D.C. were included in the study (Coates *et al.*, 1963). The authors reported that irrespective of the year of hospitalisation, infants developed comparable neutralising antibody responses to both the Long and CH 18537 strains of RSV (Coates *et al.*, 1963). This result was surprising since it had been expected that infants hospitalised in the 1956-57 season would have had a greater response to the Long strain, which was isolated in the same year and from the same city relative to the CH 18537 strain which was isolated 5 years later from a different city. It was also expected that sera from infants hospitalised in Washington D.C. in 1962 would neutralise the CH 18537 strain to a greater extent than the Long strain for similar reasons.

Wulff *et al.* looked at the ability of both rabbit hyperimmune sera and infant convalescent sera to neutralise the Long strain as well as the *"87"* strain (RSV B) isolated in Kansas in 1962 (Wulff *et al.*, 1964). The results of the study showed that rabbit anti-Long hyperimmune sera had a greater neutralising titre to the Long relative to the *"87"* strain, indicating that they were antigenically distinct (Wulff *et al.*, 1964). In contrast, infant sera obtained following natural infection in Kansas in 1962, recognised the Long strain to the same extent as the *"87"* strain, suggesting antigenic homogeneity (Wulff *et al.*, 1964). These results were therefore in general agreement with the findings of Coates *et al.* (Coates *et al.*, 1963). Similar results were reported when sera from infants in Japan were reacted against both the prototype Long strain and locally circulating Japanese strains (Suto *et al.*, 1965). Despite evidence of antigenic heterogeneity between some of the Japanese strains and the Long strain (following reaction with hyperimmune guinea pig sera), infant sera neutralised the Long strain to the same extent as the local Japanese strains (Suto *et al.*, 1965), supporting previous findings. Subsequent studies reported evidence that showed that antigenic heterogeneity was not a requirement for natural re-infection. Beem *et al.* found that strains that caused repeat infections in infants and children were not antigenically different (Beem, 1967). Later work found some evidence of group-specificity of the infant RSV neutralising antibody response against either RSV A or RSV B but not to both (Muelenaer *et al.*, 1991, Roca *et al.*, 2003, Hendry *et al.*, 1988). To date no study that has systematically sought to address the group and strain-specificity of the immune response has reported a significantly greater homologous to heterologous infant neutralising response at both the group and strain/genotype level for both RSV A and B.

The paradox of lack of cross-reactive immune response and population level competition between RSV A and B

Epidemiological surveillance studies have documented evidence of population level competition between RSV A and B, as earlier discussed. The lack of strong evidence supporting the notion of group-specificity of the convalescent human response presents a challenge in explaining the population level competition between RSV A and B. The regular and predictable sequence of alternation in transmission between RSV A and B, seen in some studies, is strongly suggestive of an underlying selection mechanism. The most plausible explanation for this cyclic alternation in dominance is population level immune selection of the dominant strain. It is reasonable to hypothesize that cyclic alternation of transmission of RSV A or B can be explained by the generation of population level group-specific immunity which precludes, albeit transiently, the continued transmission of a particular group in the population. A high level of population level group-specific immunity (relative to the alternative group) would then reduce the likelihood of re-introduction of the most recently predominant group in the population in the subsequent RSV season while giving a transmission advantage to the alternative group.

Closer scrutiny of the early cross neutralisation work reveals a number of important study design problems that could potentially have confounded interpretation of the results. At the time the studies were being conducted, the technology to definitively characterise each individual infecting strain did not exist. With the advent of monoclonal antibodies and sensitive molecular genotyping techniques such as the polymerase chain reaction (PCR), gene sequencing and restriction fragment length polymorphism (RFLP) analysis, detailed molecular characterisation of viruses infecting individual patients became possible. Without these data, it would have been impossible to ascertain that the infecting group or genotype

was identical for all infants recruited in from particular RSV transmission season, a key assumption of the early studies. If, for example, infants were recruited in an epidemic where RSV A and B were co-dominant (as has since been reported in a number of surveillance studies (Hendry *et al.*, 1989, Freymuth *et al.*, 1991)), there would have been equal probability of infection with either RSV A or B, fundamentally invalidating the presumption of homotypic exposure in a single epidemic and thereby confounding interpretation of the data. In the event that both RSV A and B infected infants were recruited, proper interpretation of the data would have been difficult. Further, many of the early studies utilised small sample sizes in their analyses. For example the study by Coates *et al.* (Coates *et al.*, 1963) utilised a sample of only 15 infants while that by Wulff *et al.* (Wulff *et al.*, 1964) used a sample of 18 infants. It is possible the small samples sizes may have obscured the detection of a small but biologically significant difference between homologous and heterologous neutralising antibody responses.

Comparison of the F and G gene sequences of RSV A and B

The availability of sequence data has enabled direct comparison at the genetic level of the differences between RSV A and B. Johnson *et al.* sequenced the G gene mRNAs of representative RSV A and B strains, Long (RSV A) and CH 18537 (RSV B). They then compared these sequences (and their deduced amino acid sequences) to the G gene sequence of another RSV A strain A2 (Johnson, 1987). When the Long G mRNA sequence was compared to the A2 G gene sequence, the authors found that they shared 94% amino acid identity. In contrast, the CH 18537 strain only shared 53% amino acid identity with the A2 G. The authors found that most of the variation was in the extracellular domain of the G protein (Johnson *et al.*, 1987b). Sullender *et al.* investigated G gene sequence variation between the A2 strain and the 8/60 strain (RSV B). They reported 56% amino acid identity between the RSV G genes of A2 and 8/60 (Sullender *et al.*, 1990). They further reported that the 8/60 G

shared 98% amino acid identity with the CH 18537 strain (Sullender *et al.*, 1990). These results were in general agreement with the G gene variability estimates reported by Johnson *et al.* (Johnson *et al.*, 1987b). Cane *et al.* undertook a more detailed analysis of the nature of the variation in the extracellular domain of the G protein. They reported that variation in this extracellular domain was not evenly distributed across the length of the protein. An extensive hypervariable domain on the carboxy terminus of the protein was separated from a less variable domain on the amino terminus by a highly conserved region (Cane *et al.*, 1991).

Johnson *et al.* studied the degree of relatedness between the F protein sequences of representative RSV A and B strains (Johnson and Collins, 1988). They reported that there was overall 91% identity between the deduced amino acid sequences of strains A2 and CH 18537 (Johnson and Collins, 1988). Viewed together, these data present an apparent paradox – of the two proteins that are targets of neutralising antibodies, one shows extensive sequence divergence between groups (G protein), while the other appears to be highly conserved between groups (F protein). Assuming that diversity in both proteins is driven by host immune pressure it is surprising that the F protein, which is the major target of neutralising antibody immunity, would be under less selection pressure relative to the G protein. An alternative explanation for the greater diversity on the G protein relative to the F is that the F protein could be subject to greater functional constraint reducing the level of variation that could be viably tolerated. However, the notion of functional constraint in a major neutralising target fails to accord with the well documented ability of RSV to re-infect at relatively regular intervals (Hall *et al.*, 1991). It appears therefore that relative sequence homogeneity in a major neutralising antibody target does not necessarily correlate with protection from re-infection. It is possible that neutralising antibodies target only a small but functionally important part of the protein and it is these antibodies that drive the limited

variability within the F protein. There is some evidence that the variable regions of the F protein coincide with known neutralising and Cytotoxic T Lymphocyte (CTL) epitopes (Agenbach *et al.*, 2005), suggesting that despite extensive sequence conservation on the F protein, protective immune responses may be directed at variable parts of the protein.

Analysis of antigenic similarity between RSV A and B

Estimates of genetic relatedness between specific genes from the two groups of RSV provide little insight on the effect of variation on the neutralising antibody response. If amino acid variation does not occur within neutralising/protective epitopes, its impact on the protective response is likely to be limited. In order to obtain an estimate of how the observed inter group genetic variability is related to protective immunity, Johnson *et al.* intranasally infected different groups of cotton rats with different strains of RSV A and B and assessed the degree of cross reactivity in their respective hyperimmune sera (Johnson *et al.*, 1987a). In order to estimate the degree of antigenic relatedness between RSV A and B, they used the Archetti-Horsfall formula (Archetti and Horsfall, 1950) – whose result is a product of two quotients, each obtained by dividing the heterologous titre by the homologous titre for each of the two strains being compared. Thus in order to infer the degree of antigenic relatedness between the Long and CH 18537 strains, the heterologous response to the CH 18537 strain (i.e. neutralisation of strain CH 18537 by Long-specific hyperimmune sera) is divided by the homologous response to the Long strain (i.e. neutralisation of the Long strain by Long-specific hyperimmune sera) and this quotient is then multiplied by the quotient obtained by dividing the heterologous response to the Long strain by the homologous response to the CH 18537 strain. The result, which is multiplied by 100, is the percent antigenic relatedness between the 2 strains. The authors reported 25% antigenic relatedness between the Long and CH 18537 strains and 28% antigenic relatedness between the A2 and CH 18537 strains (Johnson *et al.*, 1987a). A potential problem with the approach taken in this study is that the

estimates are derived on the basis of animal responses, which may be different from human responses as suggested by the early neutralisation work. To address this concern, studies to estimate the degree of antigenic relatedness using human convalescent sera obtained from infants with primary RSV A and B were conducted by Hendry *et al.* (Hendry *et al.*, 1988). An antigenic relatedness estimate of 31% between RSV A and B was calculated using the Archetti-Horsfall formula (Hendry *et al.*, 1988). It is notable that this estimate was comparable to the estimates obtained from animal studies of between 25% and 28%. These data therefore suggest that the infant neutralising response to RSV is not significantly different from that obtained from experimentally infected animal models. This conclusion is at odds with the results of the cross neutralisation studies of the 1960s that showed that showed no difference between the homologous and heterologous response (Coates *et al.*, 1963, Wulff *et al.*, 1964). It is worth noting that the infecting antigenic groups of all infants included in the study by Hendry *et al.* were determined by use of group-specific monoclonal antibodies (Hendry *et al.*, 1988).

Molecular evolution of RSV

A comparison of the deduced G protein amino acid sequences of strains isolated in the 1960s and those isolated in the late 1980s has shown that the rate of non-synonymous amino acid substitution is greater than that of synonymous substitution (Cane *et al.*, 1991), suggesting that the G protein is under positive selection pressure. It is estimated that the rate of accumulation of amino acid change over the length of the protein is 0.25% per year (Cane and Pringle, 1995). The rate of evolution of RSV can be deduced from studies of the most common recent ancestor (MRCA) of circulating strains. The MRCA of currently circulating RSV A strains has been estimated to be the early 1940s (Cane and Pringle, 1995, Zlateva *et al.*, 2004) with an average evolutionary rate of 1.83×10^{-3} nucleotide substitutions per site per

year (Zlateva *et al.*, 2004). The rate of evolution of the RSV B G gene has been estimated to be 1.95 x 10^{-3} nucleotide substitutions per site per year with the most recent common ancestor of current RSV B strains being estimated to have occurred between 1938 and 1955 (Zlateva *et al.*, 2005).

At least 6 positively selected sites have been identified on the G genes of both RSV A and B (Woelk and Holmes, 2001). There appears to be an association between the positions of the positively selected sites and known antibody epitopes (Woelk and Holmes, 2001), suggesting that immune pressure may be driving the evolution of RSV and is key to its endemic maintenance. There is however anecdotal evidence that suggests that immune selection may not be the key mechanism that drives diversity and maintenance of the virus in the population. A study of the molecular characteristics of 23 Cuban strains isolated in 1994-1995 showed that they differed from the prototypical Long strain (isolated in the United States in 1956) by only 5 amino acids (Valdes *et al.*, 1998). These data clearly conflict with the theory that immune selection is an important driver of persistence of the virus in the population. It is possible that the relative invariance of Cuban strains may be attributed to long-term political isolation that significantly reduced interaction between Cubans and the rest of the world, thereby reducing the likelihood of importation of novel strains. If this is in fact the case, the implication remains that immune selection is not a key mechanism for generation of RSV diversity and persistence of the virus in the population and that the main source of diversity may be through importation of novel strains from different populations.

The model of dissemination of different RSV strains around the world has been the focus of recent molecular epidemiology studies. The recent emergence of a unique strain of RSV B with a 60 nucleotide duplication – the BA genetic change - (Trento *et al.*, 2003) in the

hypervariable region of the G gene has provided a convenient tag with which to track both the global transmission and the accumulation of natural variation in a single strain of the virus. The first RSV B strain with a 60 nucleotide duplication (BA strain) was identified in Buenos Aires, Argentina in 1998 (Trento *et al.*, 2003). Subsequent surveillance identified this strain in Leuven Belgium between 1999 and 2000 (Zlateva *et al.*, 2005), in Birmingham United Kingdom between 2000 and 2001 (Cane, 2007), Sapporo Japan in 2000 (Sato *et al.*, 2005), in Kilifi Kenya in 2003 (Scott *et al.*, 2004), in New Delhi India between 2004 and 2006 (Parveen *et al.*, 2006). This strain has since out-competed previous strains of RSV B and as of 2012 is the most prevalent RSV B strain globally, as depicted in figure 2.7. The epidemiological success of this strain suggests that BA strains have a fitness advantage or other selection advantage over previous RSV B strains, allowing them to rapidly out-compete them globally over a relatively short period of time. The nature of this advantage is as yet unknown.

There are some data that suggest that RSV strains may become extinct with time. In an analysis of the genetic relatedness of RSV strains isolated from different parts of the world between 1956 and 1993, Cane and Pringle identified a set of RSV A isolates obtained from Northern Europe in the 1970s, which clustered distinctly in a phylogenetic analysis of the G gene (Cane and Pringle, 1995). Extensive analysis of RSV A sequences from the 1970s onwards has failed to identify similar isolates, strongly suggesting that these viruses have become extinct (Cane and Pringle, 1995, Cane, 2007). The mechanism through which extinction of strains occurs remains unknown.

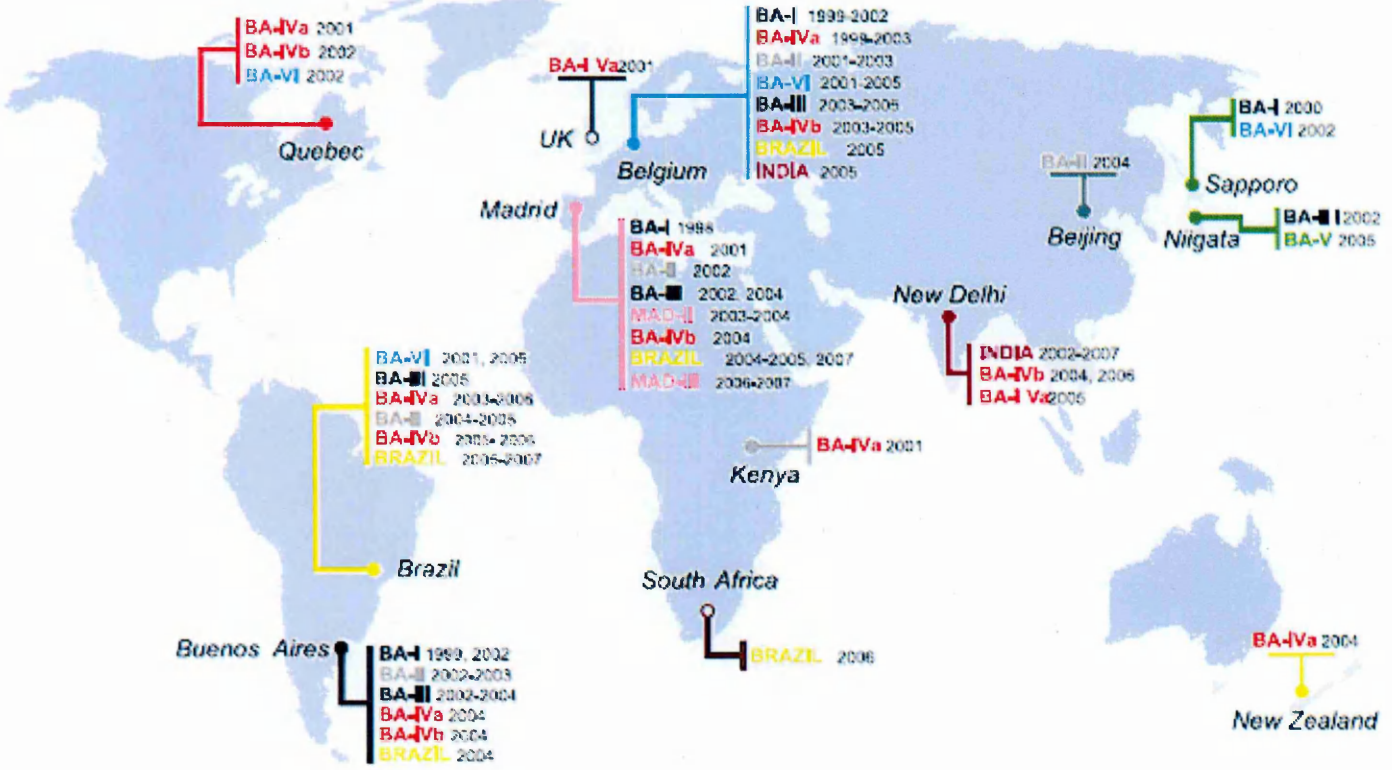

Figure 2.7 The global dissemination of RSV B strains containing a 60 nucleotide duplication in the G gene as of 2010 (Trento *et al.*, 2010).

Antigenic evolution of the F protein under immunoprohylactic pressure

There is some indirect evidence that the F protein may be under antibody selection pressure. The widespread application of immunoprophylaxis provides an opportunity to speculate on the likely model of the antigenic evolution of the F protein under antibody pressure. Palivizumab is a humanised monoclonal antibody that has been licensed for immunoprophylactic use in high risk infants (The IMpact-RSV Study Group, 1998). Palivizumab is a potent neutralising antibody that binds to a conserved region of the F protein (Beeler and van Wyke Coelingh, 1989). *In vitro* selection studies using palivizumab have shown that some single amino acid substitutions (N268S, N262S, S275F/L and K272N/M/T/Q) result in generation of neutralisation resistant escape mutants (Crowe *et al.*, 1998, Zhao *et al.*, 2004, Zhao *et al.*, 2006, Zhu *et al.*, 2011), some of which have been found to be of greater replication fitness relative to the A2 strain in competition assays (Zhao *et al.*, 2006). A number of studies have demonstrated that selection for escape mutants against this immunoprohylactic antibody can occur under natural infection conditions (Papenburg *et al.*, 2012, Zhu *et al.*, 2011). These palivizumab neutralisation resistant mutants have been identified among infants hospitalised with severe natural RSV infections and who previously were under palivizumab immunoprophylaxis (Papenburg *et al.*, 2012, Zhu *et al.*, 2011), suggesting that it can reasonably be speculated that such selection is possible with naturally produced antibodies. However the question of whether these selected mutants are transmissible and of comparable fitness with unselected wild-type strains is unknown.

Correlates of immunity from severe disease

Association between age and immune function

There appears to be an age related risk for development of severe disease, with infants under the age of one year being at greatest risk of developing severe disease. This risk appears to progressively decrease with age (Glezen *et al.*, 1986, Nokes *et al.*, 2008, Broor *et al.*, 2007, Robertson *et al.*, 2004), suggesting that as individuals grow older, they gradually develop natural resistance against severe disease, while remaining susceptible to repeated infection (Hall *et al.*, 1991). The risk of severe lower respiratory infection in immuno-competent adults is relatively low, with disease primarily being manifested as an upper respiratory infection which self-resolves within an average of 10 days (Breese Hall *et al.*, 2001, O'Shea *et al.*, 2005). However in recent years RSV has become increasingly recognised as a cause of severe respiratory illness among elderly adults (Falsey *et al.*, 2005), an outcome that has been partly attributed to age-related defects in the cellular immune function (Lee *et al.*, 2005, Liu and Kimura, 2007). The risk of severe RSV is also elevated among immuno-suppressed organ transplant patients who are typically under heavy immunosuppressive therapy to minimise the risk of organ rejection (Ison, 2009). In all three instances increased severity of disease may point to underlying immunological deficiencies. In the case of infants and elderly adults, severity may be associated with immunological immaturity and senescence respectively and in immuno-suppressed adults, severe disease may be associated with the depletion of both cellular and humoral responses by immunosuppressive drugs.

Physiological maturity of the lung

The lung pathology associated with acute bronchiolitis caused by RSV in infants is characterised by necrosis of virus infected cells in bronchial epithelia (Aherne *et al.*, 1970, Johnson *et al.*, 2007). Along with increased mucus secretion and a lymphocytic infiltrate, these cells form a dense plug which occludes smaller bronchioles, impairing respiratory function (Aherne *et al.*, 1970). The reduction in incidence of severe disease with age suggests that anatomical and physiological maturation may confer some resistance from severe illness. Between infancy and adulthood there is up to a 2.5 fold increase in airway size and alveoli count (Dunnill, 1962), suggesting that age-associated physiological changes may influence clinical outcome as a result of the increased capacity of the airways. This increased capacity may play a role in modulating severity in older individuals by providing reserve capacity through which some degree of normal respiration can continue even in the presence of cellular debris in the airways brought about by RSV infection.

Protective antibody responses

Protection from infection and disease appears to correlate strongly with the presence of neutralising antibodies. Maternal antibodies have been associated with protection from severe RSV disease in infants (Glezen *et al.*, 1981a). However, maternal antibodies have also been suggested to have a role in limiting the infants' native response to infection (Parrott *et al.*, 1973, Brandenburg *et al.*, 1997). The role of maternal antibody in preventing severe disease has been disputed in a study of the risk factors for severe RSV infection among Alaskan children, which failed to find a protective association between maternal neutralising antibodies and protection from hospitalisation (Bulkow *et al.*, 2002). The results of this study have however been

controversial as a result of potential inequality of exposure between cases and controls that may have confounded data interpretation (Munoz and Glezen, 2003).

Administration of high doses of RSV specific intravenous immune globulin (RSVIG) has been associated with a reduced risk of severe RSV disease in high risk infants and children (Groothuis *et al.*, 1993). The protective role of antibodies in preventing serious illness among infants has been further underscored by use of prophylactic immunotherapy. Palivizumab, a humanised monoclonal antibody which has been licensed for prophylactic use in pre-term and other high risk infants significantly reduces the risk of developing severe RSV infection (The IMpact-RSV Study Group, 1998). Some studies have defined a minimum protective neutralising antibody threshold for protection against severe infection. Piedra *et al.* defined a minimum threshold of 6.0 $\log_2$ PRNT and 8.0 $\log_2$ Plaque Reduction Neutralisation Titre (PRNT) as protective against hospitalisation with RSV A and B respectively (Piedra *et al.*, 2003). However the reliability of these estimates in predicting protection may be confounded by other factors that could independently account for protection such as gestational age and age of the infant.

Among elderly adults, antibodies are similarly associated with protection from severe illness. Serum IgG and nasal IgA to the F and G proteins as well as serum neutralising antibody have been found to be at lower titres in RSV infected adults compared to matched controls (Walsh and Falsey, 2004). Low titres of neutralising antibodies have also been associated with the risk of hospitalisation among adults (Walsh *et al.*, 2004).

Duration of antibody responses to RSV

The duration of both maternal and acquired antibody responses is an important factor for protection. The infant maternal antibody half-life is estimated to be of the order of less than three months (Ochola *et al.*, 2009, Ogilvie *et al.*, 1981). The infant serum IgM and IgG response to primary infection declines to pre-infection levels within a year (Welliver *et al.*, 1980). The secondary response is of greater magnitude than the primary response and potentially lasts for a longer duration of time (Welliver *et al.*, 1980). On the other hand, the nasal primary IgA, IgM and IgG response to infection all appear to decline to pre-infection levels by 3 months post-infection (Kaul *et al.*, 1981). Conversely, the rate of development, titre and duration of secondary nasal IgA, IgM and IgG responses appears to be greater than that of the primary response (Kaul *et al.*, 1981). These data suggest that both the acquired secretory and humoral responses to RSV are short-lived and may be related to the ability of the virus to repeatedly re-infect.

The ability of antibodies to protect not only from severe disease but from infection can be gleaned from experimental human infection studies. In a number of such studies, the ability of the virus to successfully infect adult volunteers has been shown to be inversely related to the pre-inoculation serum neutralising antibody titre (Hall *et al.*, 1991, Lee *et al.*, 2004, Mills *et al.*, 1971). These data suggest that antibodies probably have a dual role in protection; (i) against infection in the first instance and (ii) limiting virus infectivity upon infection. However the short-lived duration of antibody responses to RSV (Welliver *et al.*, 1980, Kaul *et al.*, 1981) suggests that such protection is likely to be transient.

Innate immune response to RSV infection

Cytokines are an important element of the early immune response to RSV. RSV infection elicits production of an array of cytokines that mediate a number of functions that are not only necessary for virus clearance but that may also promote pathology. T cells produce pro-inflammatory cytokines/chemokines in response to RSV infection. Type 1 T helper (Th1) cells produce IFN-γ while Th2 cells produce IL-4, -5, -6 and -13. The type of T helper response elicited in response to re-infection is largely dependant on the cytokine milieu present at the time of priming (Openshaw, 2002). An imbalance in Th1/Th2 responses to RSV has been cited as a contributor to severe illness (Folkerts *et al.*, 1998). RSV associated disease severity appears to be the product of a Th2 skewed response (Becker, 2006) although this has not been consistently confirmed in the respiratory secretions of infants with acute RSV bronchiolitis (Garofalo *et al.*, 2001).

Respiratory Syncytial Virus Vaccines

The goal of developing an effective vaccine has been hindered by a number of factors. The peak of disease severity in the paediatric population occurs among infants who are less than 3 months old (Glezen *et al.*, 1986) and who have a high titre of maternal antibody (Murphy *et al.*, 1986a). Vaccination at this age faces a number of important potential challenges. The response to vaccination may be inadequate due to immunological immaturity – younger infants have up to a 10-fold lower convalescent phase response to the G and F protein as well as the neutralising response compared to older infants (Murphy *et al.*, 1986b). A limited response to vaccination at this age may also be the result of suppression of the infant response by maternal antibodies.

Evidence of this may be gleaned from studies that shown that the magnitude of the convalescent infant antibody response is inversely related to acute phase serum antibody levels (Parrott *et al.*, 1973). It is also possible that the success of any future vaccine may depend on its antigenic coverage - monovalent vaccines based on strains from one antigenic group may provide insufficient protection from wild-type challenge with a diverse range of strains. Two vaccine candidates have been extensively characterised over the years are the formalin-inactivated (FI) vaccines and live attenuated vaccines. The course of development of these vaccines and their postulated mechanisms of action are discussed below.

The formalin-inactivated vaccine

Following the successful development of other formalin-inactivated vaccines such as the poliovirus vaccine in the 1950s studies of formalin-inactivated (FI) RSV vaccines were conducted in the United States in the mid to late 1960s. In a study carried out in 1965, Potash *et al.* used a formalin-inactivated RSV (strain MK5) vaccine concentrated 25-fold by alum precipitation to test for safety and immunogenicity. Vaccinated guinea pigs developed high titres of post-vaccination serum neutralising antibodies (Potash *et al.*, 1966). In the clinical arm of the study, children and adults inoculated intramuscularly with the FI vaccine developed modest serum neutralising antibodies and did not exhibit any severe vaccine-related adverse effects for up to 10 days after vaccination (Potash *et al.*, 1966). It was thus concluded that the vaccine was safe with no systemic adverse effects associated with vaccination (Potash *et al.*, 1966). In 1966 two large scale clinical trials of the FI vaccine in infants and children were carried out. The first, carried out between September and December 1966, recruited infants and children between 4 months and 10 years of age to whom two 0.5

ml intramuscular doses of 100-fold concentrated formalin-inactivated RSV (Bernett strain) vaccine was administered (Chin *et al.*, 1969). A control arm was included in the study for the purpose of comparison. The control arm received a trivalent parainfluenza vaccine containing parainfluenza 1, 2 and 3. In total 191 children received the full dose of FI vaccine while 194 children received the full dose of the control parainfluenza vaccine (Chin *et al.*, 1969). 68% of the FI vaccinees had a 4-fold or greater rise in antibodies against RSV in their post-vaccination sera, while only 0.9% of the control parainfluenza vaccinees had a 4-fold or greater rise in anti RSV titre (Chin *et al.*, 1969). However in the subsequent RSV season, the incidence of medically attended RSV infection in infants less than 1 year of age in the FI vaccine group was approximately 3 times greater than the control group. Overall, the incidence of severe disease in the FI vaccine group across all ages (7.9%) was almost double that in the control group (4.7%) (Chin *et al.*, 1969). 60% of the FI vaccinees who got natural RSV infection were hospitalised compared to 22% of the control group vaccinees who were hospitalised following natural RSV infection (Chin *et al.*, 1969). The severity of disease among hospitalised infants from the FI vaccine group was much greater than that of infants in the control group; 44% of the hospitalised infants in the FI vaccine group had severe or very severe pneumonia compared with 5.6% among the controls (Chin *et al.*, 1969). A second clinical trial of the FI vaccine was conducted between December 1965 and December 1966 where infants between 2 and 7 months of age were recruited (Kim *et al.*, 1969). A similar vaccine formulation and regimen as in the study described above was administered. Similarly a parainfluenza vaccine control group was included in the study. The results of this trial showed that post-vaccination neutralisation titres to RSV were 6-fold greater in the FI vaccine group compared to the control group (Kim *et al.*, 1969). However despite

serological evidence of comparable exposure between the two groups in the subsequent RSV season, 80% of FI vaccinees in this study required hospitalisation following natural infection (Kim *et al.*, 1969). In contrast only 5% of infants in the control group who experienced natural RSV infection required hospitalisation. Also, the severity of illness was greater among the FI vaccinees relative to the control group (Kim *et al.*, 1969). Tragically, two toddlers aged 14 and 16 months who were in the FI vaccine group died upon natural exposure to RSV. The two infants had received the first of 3 inoculations of FI vaccine at 2 and 5 months respectively. Post-mortem examinations found evidence of extensive bronchopneumonia, pneumothorax and eosinophilia (Kim *et al.*, 1969).

Postulated models of FI vaccine mediated pathology

The failure of the FI vaccine led to studies to investigate why the vaccine potentiated disease upon natural exposure. Using post-vaccination sera from infants and young children who had received the FI vaccine, Murphy *et al.* found that young infants developed a high antibody titre to the F protein but had poor response to the G protein (Murphy *et al.*, 1986c). In contrast, older infants and young children developed high titres to both F and G proteins. None of the vaccinated infants and children developed neutralising antibody titres comparable to that of age-matched individuals who had undergone natural infection (Murphy *et al.*, 1986c). It was concluded that formalin inactivation had somehow altered epitopes on the F and G proteins resulting in the development of non-functional (non-neutralising) antibodies. The authors postulated 3 models in which these non-neutralising antibodies could have potentiated disease; (i) through the formation of immune complexes in the lung, (ii) a poorly developed anti-G response in young infants could have reduced protection from subsequent

challenge and (iii) the poor neutralising response could have delayed the development of effective responses to clear the virus (Murphy *et al.*, 1986c). Subsequent studies found that in addition to the poorly neutralising response, F protein specific antibodies to the FI vaccine were deficient in fusion inhibiting activity, promoting the spread of the virus in the respiratory tract upon natural infection (Murphy and Walsh, 1988). Later work suggested that the failure to develop an effective neutralising response following FI vaccination was not due to formalin disruption of neutralising epitopes but rather due to the development of low avidity anti-virus antibodies due to the lack of affinity maturation (Delgado *et al.*, 2009). This interpretation has however been disputed (Shaw *et al.*, 2009). A model of immune mediated disease exacerbation has been proposed based on work with animals. Connors *et al.* found that in the absence of CD4+ and CD8+ T cells, antibodies are unable to mediate disease enhancement (Connors *et al.*, 1992). Later work found that mice vaccinated with FI vaccines had a marked increase in type 2 CD4+ helper T cell cytokines and lower type 1 T helper cell responses (Waris *et al.*, 1996). Formalin treatment of RSV antigens was later shown to promote the development of type 2 responses (Moghaddam *et al.*, 2006). These data suggested that FI vaccine mediated disease exacerbation may have been the result of an over exuberant inflammatory response to infection.

Development of live attenuated RSV vaccines

Following the failure of the FI RSV vaccines of the 1960s attention shifted to the development of live attenuated vaccines. Live attenuated RSV vaccines were first developed through extensive serial passaging of the A2 strain at progressively lower temperatures i.e. cold passaging or *cp* (Friedewald *et al.*, 1968). Adaptation of a live,

intranasally delivered vaccine for replication at lower temperatures is considered important since the temperature at the upper respiratory tract is lower than the core body temperature (Polack and Karron, 2004). Experimental infection of adult volunteers showed that one such virus that could grow at 26°C had lost virulence in adults (Friedewald *et al.*, 1968), but had retained virulence, despite showing some evidence of immunogenicity, in infants under the age of 2 months (Kim *et al.*, 1971). To further attenuate the virus, chemical mutagenesis was used to induce a temperature sensitive (*ts*) phenotype in the *cp* vaccine strains (Kim *et al.*, 1973). Temperature sensitive mutants have a shutoff temperature above which they are unable to replicate – thus mutants with shutoff temperatures that are close to the core body temperature are unlikely to replicate in the lower respiratory tract and cause severe disease (Murata, 2009). Initial clinical trials with *cp-ts* live attenuated RSV vaccines showed that they were still insufficiently attenuated and were genetically unstable (Kim *et al.*, 1973). Subsequent studies reported the generation of *cp-ts* strains with remarkably low shutoff temperatures and greater genetic stability (Crowe *et al.*, 1994b). One such strain (*cpts*-248) had a shutoff temperature of 38°C and was highly attenuated and immunogenic in animal studies (Crowe *et al.*, 1994b). Further attenuation of the *cpts*-248 mutant was done through chemical mutagenesis, resulting in a highly attenuated daughter strain *cpts*-248/404 with a shutoff temperature of 36°C and with up to 1000-fold reduction in its ability to replicate *in vivo* in animal models (Crowe *et al.*, 1994a). A live attenuated vaccine based on the *cpts*-248/404 mutant was tested in infants where some evidence for protection from disease following natural infection was reported (Wright *et al.*, 2000). Nonetheless, the vaccine was considered to be insufficiently attenuated as it caused upper respiratory tract congestion in young infants (Wright *et al.*, 2000), a potentially fatal problem for young infants who are

obligatory nose breathers (Bergeson and Shaw, 2001). The vaccine was however considered to be sufficiently attenuated for older children (Wright *et al.*, 2000). Further attenuation through reverse genetics, resulted in a vaccine candidate that was sufficiently attenuated for young infants but that insufficiently elicited antibody responses (Karron *et al.*, 2005).

Potential for indirect protection of vulnerable infants using live attenuated vaccines.

As described above, there are currently available live attenuated vaccines that appear to provide some protection upon natural exposure and are safe for use in young children. Safety concerns however preclude the use of these vaccines in the paediatric infant population who are at the greatest risk of severe disease (Wright *et al.*, 2000). In the absence of a sufficiently attenuated and immunogenic vaccine for the youngest infants, the potential of using these vaccines in older age groups in order to reduce the risk of transmission to the infant is clearly worth consideration. Maternal vaccination is a potential route through which these vaccines could be given in order to boost the titre of trans-placentally transferred antibodies – and hopefully delay the age at which infants first become susceptible to natural infection. The maternal vaccination model for infant protection is currently being used for other infectious diseases. Maternal vaccination with influenza virus vaccine has been reported to result in significant reduction in the number of neonatal hospitalisations with influenza (Poehling *et al.*, 2011, Benowitz *et al.*, 2010).

Chapter 3 – Materials and methods

Overview

This chapter will provide a detailed description of both the participants and the laboratory methods used in this study. The first part of the chapter will describe the study site as well as general characteristics of the study participants. The studies that are presented in this thesis were nested within three previously established studies, i.e. the Kilifi District Hospital (KDH) inpatient RSV surveillance study, the Kilifi Birth Cohort (KBC) study and the household RSV transmission study. The structure of these studies will be described in detail in the first part of this chapter.

The second part of this chapter will provide a detailed description of the laboratory assays used in this study. A detailed description of the following assays and techniques will be provided: cell and virus culture, plaque assay, immunofluorescent Antibody Testing (IFAT), measurement of cytokine/chemokine concentrations in nasal samples using the MSD mesoscale platform, RNA extraction, cDNA synthesis, polymerase chain reaction (PCR) and gene sequencing on the ABI 3130xl platform.

Study site, Population and Sampling

This study was conducted in Kilifi, a rural African district located on the Kenyan Coast. Kilifi experiences high humidity all year round, with two annual rainy seasons; April-July and November-December (Nokes *et al.*, 2009). Although incidence has declined in recent years, the area has traditionally been endemic for malaria, with its associated high morbidity and mortality burden. The studies described here were carried out at the Kilifi District Hospital (KDH) which provides both adult and

paediatric inpatient and outpatient care. As mentioned earlier, the nasal and serum samples used in the study were derived from three established studies. Below is a detailed description of these studies.

The RSV Inpatient Surveillance study at KDH

The samples that were derived from the inpatient surveillance study for use in the present study were obtained from infants and young children between the ages of 1 day and 59 months. These individuals had been admitted to the KDH paediatric ward between December 2002 and February 2008, during which 6 RSV epidemics occurred in Kilifi. The infants included in this study were recruited during 3 of these 6 epidemics: the 2002/2003 epidemic, the 2005/2006 epidemic and 2007/2008 epidemic. The 2002/2003 epidemic was characterised by transmission of RSV A and B in roughly equal proportions, the 2005/2006 epidemic was dominated by RSV A transmission, while the 2007/2008 epidemic was dominated by RSV B transmission. These data are shown in figure 2.1 in Chapter 2. Infants who were admitted met the World Health Organization (WHO) criteria for the clinical syndromes of (i) *severe pneumonia*, i.e. cough or difficulty in breathing plus lower chest wall indrawing in addition to showing no signs of very severe pneumonia, or (ii) *very severe pneumonia* i.e. cough or difficulty in breathing plus at least one of the following: hypoxia, defined as an oxygen saturation of less than 90% by fingertip pulse oximetry, inability to drink, breast feed or to sit or impaired consciousness (WHO, 2005). Upon admission, nasal samples were collected for detection of RSV by immunofluorescent antibody testing (IFAT). Acute phase sera were collected at the time of admission while convalescent phase sera were collected from RSV positive infants approximately one month later. Both nasal and serum samples were stored at -80°C for later use.

The Kilifi Birth Cohort (KBC) Study

The second set of samples was derived from the Kilifi Birth Cohort (KBC) study. Participants were recruited at the maternity ward and the Maternal and Child Health Clinic (MCHC) at KDH. Infants were recruited in two phases over two calendar years. Each recruitment phase took place over approximately 6 months and the two recruitment phases were separated by approximately 6 months. The cohort was monitored for approximately four years, until each respective phase had experienced at least 3 RSV epidemics. During RSV epidemics, active surveillance visits by field workers were scheduled every week. Surveillance was also carried out if infants presented to the research outpatient clinic or if they were admitted at KDH. Home visits which identified signs of lower respiratory infection, resulted in referral to the clinic. Caregivers were asked to visit the clinic if they identified symptoms of respiratory infection in the children recruited in the study. Nasal washes were collected during home or clinic visits if in the preceding week, they had been observed to have either (i) difficulty in breathing (ii) a runny nose or nasal congestion or (ii) an acute cough. In the paediatric ward, infants identified to have either a lower respiratory tract infection, bronchiolitis or severe/very severe pneumonia had nasal wash or nasal pharyngeal aspirate samples collected. Collected nasal samples were tested for presence of RSV antigen by IFAT. If RSV was identified in nasal secretions, an acute serum sample was collected and a convalescent serum sample was thereafter collected after a period of approximately one month.

The RSV household transmission Study

This study was designed to characterise the chains of transmission of RSV within the household. Between November 2009 and June 2010, the study recruited 43 households in which all members were repeatedly sampled every 3 to 4 days (irrespective of symptoms) over the duration of an RSV epidemic for evidence of infection with RSV. A range of other common respiratory viruses was also detected. Households were eligible for recruitment if they had a child born after the previous RSV epidemic and had at least one elder sibling to the infant. A total of 19,816 home visits were conducted in which 16,284 nasal flocked swabs (NFS) were collected from 554 participants. The studies described in this thesis investigated the development cytokine/chemokine responses in serially collected nasal flocked swab samples obtained from 10 infants within the household transmission study. These infants experienced at least one natural RSV infection over the course of follow-up and their cytokine/chemokine responses before, during and after infection are reported.

Ethical Considerations

In each of the studies detailed above, written informed consent was sought from the parents and guardians of the infants and children prior to sample collection. All studies received ethical clearance from the Kenya Medical Research Institute Ethical Review Committee.

Laboratory methods

Cell and virus culture

Test viruses were initially propagated on HEp-2 cells to amplify virus titre. Cells were grown in T-25 flasks to between 70-80% confluence in a 37°C humidified CO_2 incubator. Contemporary virus strains Ken/A/2006 and Ken/B/2008, were isolated from the nasal washes of infants with acute RSV infection while historical strains A2 (Aus/A/1961) and 8/60 (Swe/B/1960) were kindly provided by Prof Pat Cane. 500µl of seed virus was diluted in 500 µl of minimum essential medium (MEM) containing 2.5% foetal calf serum (FCS) and penicillin/streptomycin (maintenance media). 1 ml of seed virus was transferred to the HEp-2 monolayer that had been washed twice with sterile Phosphate Buffered Saline (PBS). The flasks were then incubated for 2 hours at 37°C with intermittent inversion at 15 minute intervals to increase the likelihood of successful virus attachment to receptors on HEp-2 cells. The flasks were then replenished with 7ml of maintenance media and incubated for up to 5 days. The flasks were observed daily for evidence of virus mediated cytopathic effect (CPE). Once CPE was seen, the virus infected cells were dislodged from the surface of the flask and into the media. The newly established virus stocks were then aliquoted into 1 ml volumes and stored at -80°C for future use. To confirm successful virus infection, the new virus stocks were evaluated using immunofluorescent antibody testing (IFAT).

Immunofluorescent Antibody Testing (IFAT)

250 µl of new virus culture stocks were aliquoted into cytospin funnels and centrifuged in a desktop centrifuge at 1000 rpm for 10 minutes. Cytoslides were then

air dried for 30 minutes and fixed in cold acetone for 10 minutes. Following fixing, the slides were air dried for a further 5 minutes. A drop of an RSV detection reagent containing a mixture of a fluorescein isothiocyanate (FITC) conjugated anti-RSV antibody and evans blue counterstain was placed on the fixed cells on the cytoslides (Millipore). The slides were then incubated at 37°C for 30 minutes in a humidified CO_2 incubator. The slides were then washed three times in PBS and air dried for an hour. The dried slides were prepared using an oil immersion and examined at x100 magnification on a fluorescent microscope. Figure 3.1 (a) is an example of an immunofluorescent slide containing successfully cultured virus (Ken/A/2006), while figure 3.1 (b) is a negative control (HEp-2 cells).

(a) (b)

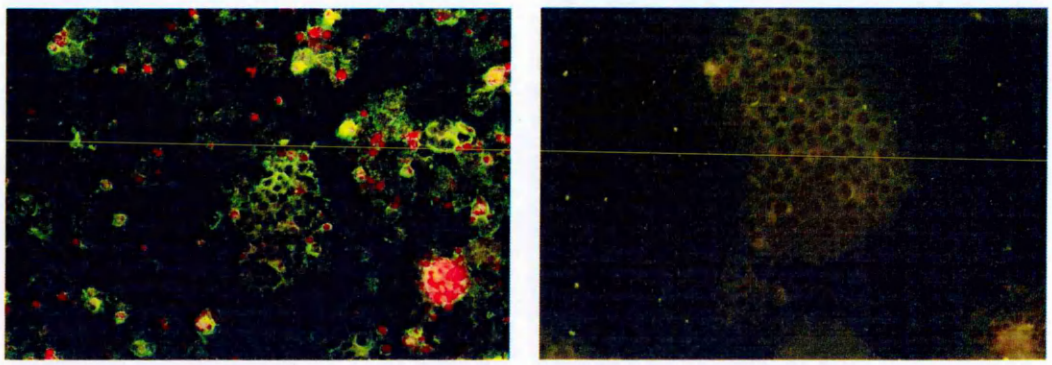

Figure 3.1 (a) Immunofluorescence (bright apple-green colour) from an IFAT slide containing HEp-2 cells which were infected with the A2 strain of RSV while (b) is a negative control containing uninfected HEp-2 cells.

Plaque assay

Virus titre was determined using the plaque assay. HEp-2 cells were seeded on 96 well cell culture plates at a concentration of 20,000 cells per well and grown

overnight to confluence at 37°C in a humidified CO_2 incubator. 10-fold dilutions of stock virus were prepared as follows for inoculation onto the HEp-2 monolayer.

Neat virus 50 µl + 450 µl MEM = 10^{-1} dilution.

10^{-1} dilution 50 µl + 450 µl MEM=10^{-2} dilution.

10^{-2} dilution 50 µl + 450 µl MEM=10^{-3} dilution.

10^{-3} dilution 50 µl + 450 µl MEM=10^{-4} dilution.

Media was removed from the cells on the 96 well plates and 50 µl of each virus dilution added to successive wells of the plate in duplicate. The plates were then incubated for 2 hours at 37°C in a humidified CO_2 incubator with intermittent agitation at 15 minute intervals. 150 µl of MEM containing 2.5% FCS was then added to each well and the plates incubated for 48 hours in a 37°C CO_2 incubator. After the 48 hour incubation, the cells were fixed with 100 µl/well methanol containing 0.5% hydrogen peroxide for 20 minutes. The plates were then washed twice with 200 µl/well of PBS. 100 µl per well of a 1/300 dilution of a mouse anti-RSV IgG (Novocastra, Leica corporation) was then added and the plates incubated at room temperature for one hour. The plates were then washed three times with 2 00µl PBS per well followed by the addition of 100 µl/well of a 1/1000 dilution of goat anti-mouse IgG with a horseradish peroxidase (HRP) tag (Dako Corporation) and one hour incubation at 37°C.

A tablet of 3-amino-9-ethylcarbazole (AEC) (Sigma) was dissolved in 6 ml of dimethyl sulphoxide (DMSO) to give a 3.3 mg/ml concentration of AEC. The development substrate was prepared by adding 600 µl of this solution to 10 ml of 20

mM sodium acetate buffer containing 16 µl hydrogen peroxide. The sodium acetate buffer was adjusted to a pH of between 5.0 and 5.5 prior to addition of the AEC solution. 100 µl of the substrate solution then added to the test wells and the plates incubated for up to one hour at room temperature or until plaques were clearly distinguishable from the cell monolayer background. The substrate solution was then removed and the plates washed twice with 200 µl/well of PBS. Plaques were then counted using either low power microscopy or by using an ELISPOT reader with optimised read settings. Figure 3.2 is an example of RSV A2 plaque morphology under low power microscopy. To determine the virus concentration in the initial virus stock, the virus dilution that resulted in between 10 to 50 plaques was determined and this was then multiplied by the dilution factor to give the stock virus concentration in plaque forming units per ml (PFU/ml). After determination of virus titre, measurement of neutralising antibody in infant sera was carried out using a plaque reduction microneutralisation assay. This assay along with the modifications undertaken to optimise its output are discussed in detail in chapter 4.

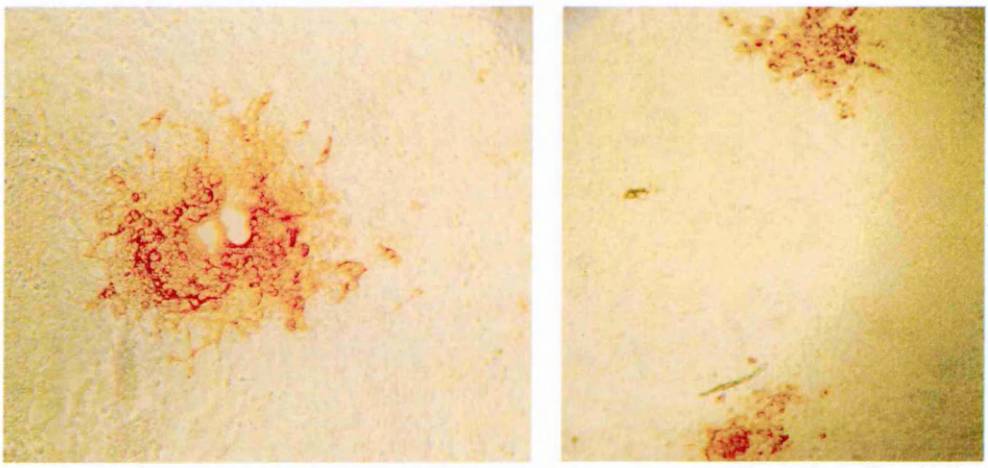

Figure 3.2 The appearance of RSV plaques on a HEp-2 monolayer background. The plaques were developed using AEC

Measurement of cytokine/chemokine concentration using the MSD mesoscale platform

The concentration of cytokines/chemokines produced following natural infection was measured using the MSD mesoscale platform (Meso Scale Discovery, Gaithersburg, Maryland, USA). The MSD platform is a proprietary system designed to measure the concentration of up to 10 cytokines/chemokines from a single sample. The assay used in this study was in the 96 well multi-array format, in which multiple cytokines/chemokines were measured in each nasal sample. The assay is designed in a sandwich immunoassay format in which capture antibodies are either coated on a single spot in one microtitre plate well, or in which multiple spots, each coated with a capture antibody specific for a different analyte are arrayed at different positions on a single microtitre plate well. The capture antibodies bind to the cytokines/chemokines of interest in the patient sample, which are in turn bound by a detection antibody that contains a proprietary tag, SULFO-TAG. This tag is activated by the working electrode that is attached to the base of each well, resulting in the production of a chemiluminescent signal, whose magnitude corresponds to the concentration of the cytokine/chemokine of interest in the sample. Figure 3.3 shows an example of a four spot array format. In this study, the MSD system was used to measure the concentrations of 10 cytokines/chemokines in the nasal secretions of infants: interleukin (IL) -6, IL1-b, Tumor Necrosis Factor (TNF)-α, Macrophage Derived Chemokine (MDC), Macrophage Inflammatory Protein (MIP)1-β, IL-8, Interferon (IFN)-γ, IL-10, IL-4 and IL-5.

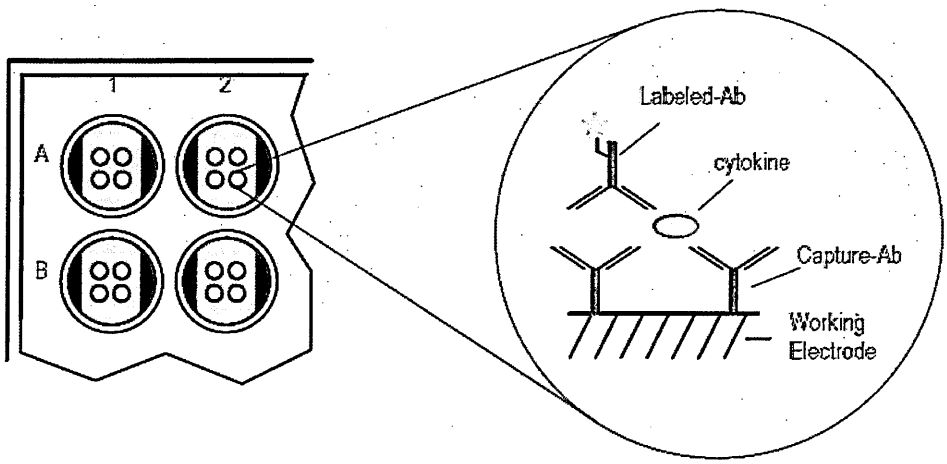

Figure 3.3 Illustration of a 4 spot MSD mesoscale plate. Each spot is coated with a capture antibody that is specific for a different analyte. These antibodies bind to the cytokines/chemokines of interest in the patient samples. The detection antibody, which is labelled using a proprietary tag (SULFO-TAG) is used to generate a chemiluminescent signal that is activated and subsequently detected by the working electrode.

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Protocol for measurement of cytokines/chemokines in the nasal secretions of infants using the MSD mesoscale system

Using a mixed calibrator solution provided by the manufacturer, four-fold standard dilutions were prepared. After preparation of the calibrator dilutions, plates were blocked by addition of 25 µl/well of a blocking solution provided by the manufacturer. The plates were then sealed and incubated for 30 minutes at room temperature on a vibrating platform (600 rpm). After blocking, 25 µl/well of either patient sample or pre-diluted calibrators were added and the plates sealed and incubated at room temperature for 2 hours on a vibrating platform. The plates were then washed three times with PBS containing 0.05% Tween 20. 25 µl/well of a detection antibody solution that was specific for the analytes of interest was added. The plates were then incubated for a further 2 hours at room temperature on a vibrating platform. The plates were then washed 3 times and 150 µl of a read buffer

provided by the manufacturer was added to each well. The plates were then read on a Sector 2400 Imager and the results analysed. Concentrations of cytokines/chemokines in individual patient samples were measured against the standard curves generated using manufacturer supplied calibrators.

Sequencing of the F and G genes of infecting RSV strains

RNA extraction and cDNA synthesis

Ribonucleic Acid (RNA) was extracted from infant nasal wash samples using the QIAamp Viral RNA mini kit (Qiagen, Germany) following the manufacturer's instructions. The buffers required for carrying out RNA extraction are proprietary and were supplied by the kit manufacturer. 560 µl of buffer AVL (cell lysis buffer) was added to 140 µl of nasal samples, vortexed and incubated for 10 minutes at room temperature to facilitate cell lysis. 560 µl of absolute ethanol was then added and the reaction vortexed prior to its transfer to a QIAamp spin column. The reaction was centrifuged at 6000 x g for 1 minute. 500 µl of buffer AW1 was then added and the reaction tube centrifuged at 6000 x g for a further 1 minute. 500 µl of buffer AW2 was then added and the spin column centrifuged at 20,000xg for 3 minutes. Elution was done by addition of 60 µl of buffer AVE. The column was thereafter incubated for 1 minute, after which, a final spin of 6000 x g was carried for 1 minute resulting in the elution of the extracted RNA into a clean collection tube. The eluted RNA was subsequently used for complementary DNA (cDNA) synthesis. cDNA synthesis was carried out using the Omniscript Reverse Transcriptase Kit (Qiagen) following the manufacturer's instructions. A 20 µl reaction mix containing reverse transcriptase buffer, deoxyribonucloside triphosphates (dNTPs), reverse transcriptase enzyme and

random primers was added to 20 µl of the extracted RNA and incubated at 37°C for 1 hour. The synthesized cDNA was stored at -80°C for subsequent PCR amplification of both the F and G genes.

G Gene amplification and sequencing

G gene amplification was carried out using a nested PCR reaction. For each reaction, 25 µl of Taq PCR Master Mix was added to 1 µl of 25 mM MgCl2, 18 µl of RNase free water, 2 µl of the forward primer AG20 (5'-GGGGCAAATGCAAACATGTCC-3') and 2 µl of the reverse primer F164 (5'-GTTATGACACTGGTATACCAACC-3'). The region amplified by these primers was between nucleotide 284 of the G gene and nucleotide 9 of the F gene. 4 µl of the cDNA sample, was then added to this reaction mix and thermocycled using the following conditions: 50° C for 30 min, 95°C for 15 min and then 40 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 1 min. A final extension of 10 min at 72°C was included. The nested PCR utilised 2 µl of the primary PCR product in the reaction mix described above. For the nested PCR, the forward primer was BG10 (5'-GCAATGATAATCTCAACCTC-3') and the reverse primer was F1 (5'-CAACTCCATTGTTATTTGCC-3'). Thermocycling conditions were as follows: 95 °C for 2 min, followed by 30 cycles of 95 °C for 45 sec, 54 °C for 45 sec, 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Successful amplification was confirmed by running the secondary PCR products on a 2% agarose gel. PCR products were purified using the QIAquick purification kit (Qiagen) following the manufacturer's instructions. Sequencing of the G gene products was done using the BigDye terminator chemistry on the 3130xl ABI instrument. For each sequencing reaction, 0.5 µl of the ready reaction premix (ABI) was added to 1.75 µl of 5X sequencing buffer, 1 µl of 5 µM primers (BG10 and F1) and 5.25 µl of water.

1.5 µl of the cleaned second round PCR product was used as the template in the reaction. Thermocyling then carried out using the following conditions: 25 cycles of 96°C for 10 seconds, 50°C for 10 seconds, and 60°C for 4 min. The sequencing extension products were then purified using ethanol precipitation. For each reaction, 8 µl of deionised water was added to the reaction tube, followed by 32 µl of 95% ethanol. The reaction tubes (on 96 well PCR plates) were then inverted several times and incubated at room temperature for 15 minutes to precipitate the sequencing extension products. The reaction vessels were then centrifuged at 2000 x g for 45 minutes after which the supernatants were discarded. The reaction vessels were inverted on paper towels and centrifuged for a further 1 minute at 700 x g. The resulting pellets were then dissolved in 3 µl of loading buffer consisting of formamide and EDTA at a ratio of 1:5. The samples were finally loaded on the ABI 3130xl genetic analyser and the sequencing reaction initiated.

F gene amplification and sequencing

RNA extraction and cDNA synthesis was carried out as described in the previous section. Amplification of the F gene product was done using similar reaction conditions as those of the first round G gene PCR and the following primers: primers FRSV-U (5'-GGCAAATAACAATGGAGTTG-3') and FRSV-4R (5'-AAGAAAGATACTGATCCTG -3'). The region amplified by these primers was between nucleotide 121 and 918 of the F gene. The thermocycling reaction adopted similar reaction conditions as the primary G gene PCR, with an annealing temperature of 52°C. The products of this PCR were purified, precipitated and sequenced as above.

Chapter 4 - Development of Plaque reduction microneutralisation assay

Introduction

Traditional plaque reduction neutralisation assays suffer from a number of critical shortcomings that have limited the widespread use of these assays. Key among these is the labour intensive element of microscopic plaque enumeration that is necessary for the determination of endpoint titres. The reliability of data obtained using this manual enumeration technique is brought into question by its inherent subjective bias. Even when carried out by the same individual, the reproducibility the results can be reasonably be assumed to vary considerably depending on many intra operator factors that vary from assay to assay. For example, operator fatigue could be argued to significantly influence the reproducibility of results.

The results presented in this thesis were primarily based on the measurement of serum neutralising antibody titres in infant sera. In order to improve the reliability of the estimates measured using this assay, a number of modifications were incorporated into the assay. This chapter will discuss the general protocol used in the neutralisation assays as well as the development of a high throughput plaque enumeration system.

Methods

Plaque Reduction Neutralisation Assay

Each test serum sample was initially diluted 1:20 in MEM and complement cascade proteins inactivated by heating the samples in a 56°C water bath for 30 minutes. The samples were then double diluted from 1:20 to 1:10,240 in a volume of 50 µl /well. Each dilution was aliquoted in duplicate. 50 pfu of test virus in a volume of 50 µl MEM was then added to the wells containing 50 µl of pre-diluted serum. The 100 µl serum/virus mix was then thoroughly mixed by repeated pipetting. Each plate contained 3 test samples and one positive control consisting of pooled adult sera to track assay reproducibility as well as a virus-only control. The serum/virus mix was incubated at 4°C for one hour to facilitate virus neutralization by serum antibodies, after which the neutralization reaction was transferred to a confluent monolayer of HEp-2 cells on 96 well plates. These plates were incubated for 2 hours at 37°C in a humidified CO_2 incubator, with intermittent mixing at 15 minute intervals to enhance the likelihood of un-neutralized virus attaching to cell surface receptors. After the 2 hour incubation, the plates were replenished with 100 µl/well of MEM containing 2.5% FCS and incubated for 48 hours in a 37°C humidified CO_2 incubator.

After 48 hours, media was removed from the cells and the plates fixed by addition of 100 µl/well methanol containing 0.5% hydrogen peroxide for 20 minutes. The plates were washed two times with 200 µl/well of PBS. 100 µl/well of a 1/300 dilution of a mouse anti RSV IgG (Novocastra, Leica Corporation) in PBS was added and the plates incubated at room temperature for 1 hour. The plates were thereafter washed 3 times with and 100 µl/well of a 1/1000 dilution of goat anti-mouse IgG with a

horseradish peroxidase (HRP) tag (Dako Corporation) added. The plates were then incubated at room temperature for an hour. After this incubation, the plates were washed three times and a substrate consisting of AEC and sodium acetate added as described in chapter 3. Plaques were counted using an ELISPOT reader with optimised read settings. A detailed description of these optimisations will be discussed in the next section. Figure 4.1 shows an example of a fully developed plate in which the plaques are clearly visible by macroscopic examination.

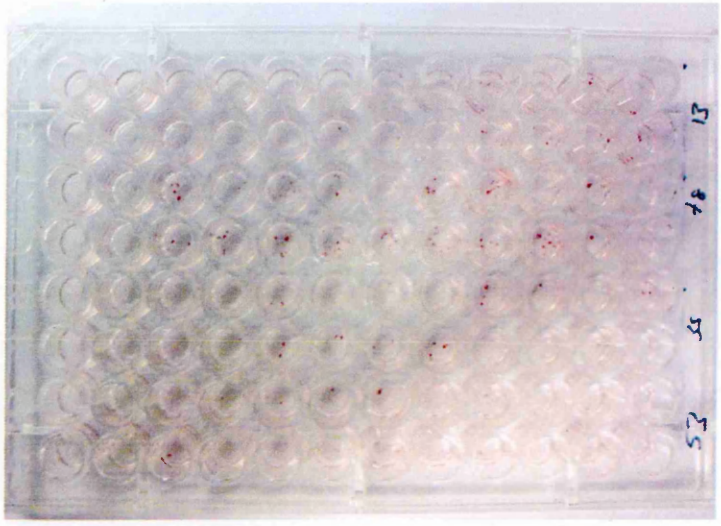


Figure 4.1 Illustration of the appearance of a plate at the completion of PRNA assay. Plaques can be clearly seen as sharp brown spots by the unaided eye.

Development of high throughput plaque counting using an ELISPOT reader

In order to increase the throughput of the neutralisation assay as well as eliminate the subjective bias associated with manual microscopic counting, an automated plaque enumeration technique was developed. This technique relied on a conventional

ELISPOT reader (AID, Autoimmun Diagnostika, Germany) for the identification and enumeration of plaques on 96 well tissue culture plates. Due to variations in the plaque phenotypes of different strains of RSV, it was necessary to optimise the read settings of each individual virus in order to increase both the sensitivity and specificity of enumeration. Figure 4.2 shows the varying plaque morphologies of the two test strains of RSV B used in this study.

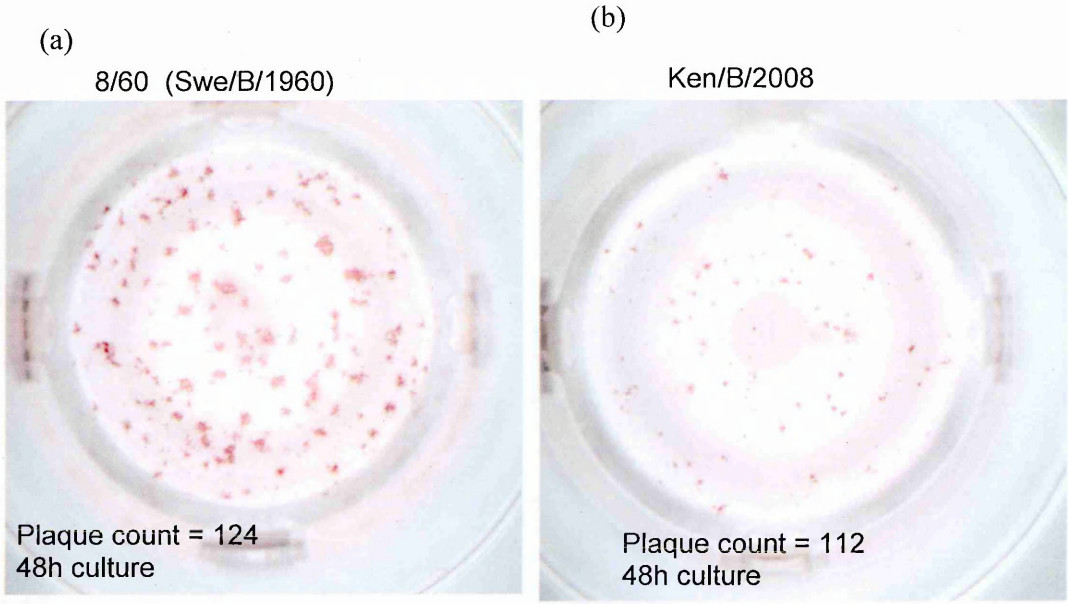


Figure 4.2 Plaque morphologies of two strains of RSV B which were grown to approximately comparable titres are shown. (a) shows the plaque morphology of the 8/60 (Swe/B/1960) while (b) shows the plaque morphology of Ken/B/2008. After a 48 hour culture, 8/60 plaques are considerably larger relative to Ken/B/2008 plaques.

Optimisation of read settings was carried out by adjusting 2 parameters: plaque size and plaque intensity. The intensity parameter was used to set the intensity (darkness) threshold for counting plaques. The value of this parameter represents the difference between the intensity of the peak of the plaque and its surrounding background. Intensity, which is measured in brightness units, took on values between 0 (White) to 255 (Black). Objects whose intensity values that fell below the set ranges were not counted as plaques. The size parameter was used to set the minimum size in pixels

that an object needed to have in order to be recognized as a plaque. Objects whose size in pixels was outside the set values for this parameter were similarly not included in the final plaque count. This parameter took on values ranging from 1 to 5000. As a result of differing plaque morphologies, optimal plaque settings were determined for each virus strain used in the study. These were the settings that resulted in a final count that most closely conformed to manual plaque counting. Once the optimal parameters had been decided, plates were placed on the ELISPOT reader and read with the optimised settings. To facilitate the automated reading, a white sheet of paper was attached to the base of each plate prior to insertion into the ELISPOT reader in order to maximise the contrast between the dark brown plaques and the clear cell monolayer background. The optimal size and intensity parameters for the different test strains used in this study are shown in table 4.1.

Virus/Setting	Size	Intensity
A2	120-5000	50-255
8/60	80-5000	35-255
Ken/A/2006	100-5000	50-255
Ken/B/2008	40-5000	35-255

Table 4.1. The optimal size and intensity and parameters of the four test viruses used in this study. These parameters produced automated counts that were most closely related to microscopy counts.

Validation of automated plaque counting using an ELISPOT reader

In order to confirm the accuracy of the ELISPOT reader plaque counting technique, parallel enumeration of plaques by both the ELISPOT reader and by microscopy was carried out. Serial 2-fold dilutions of the A2 strain were used to infect a monolayer of

HEp-2 cells from a starting titre of approximately 50 pfu/well in duplicate. Three experienced laboratory technicians were independently asked to microscopically count the number of plaques present in each well. The mean count at each dilution was then obtained for all three readers. The same plate was also subjected to enumeration by the ELISPOT reader using optimised count settings and the mean count at each dilution calculated. A comparison of the mean counts at each dilution of virus as determined by both techniques is shown in figure 4.3.

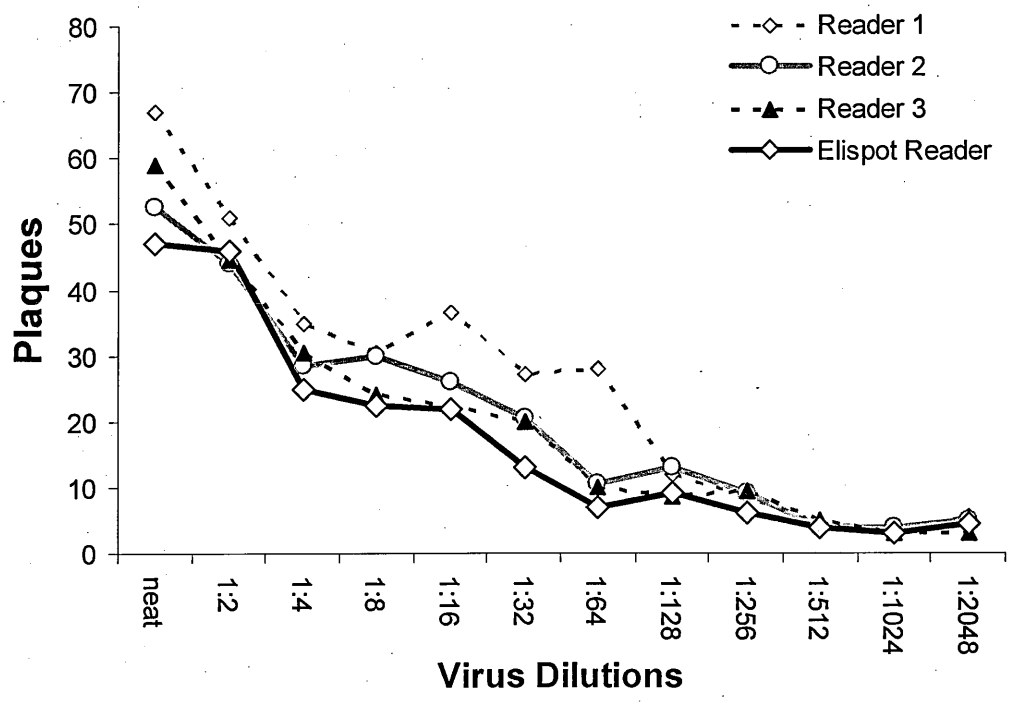


Figure 4.3 Comparison of mean plaque counts at different dilutions of virus by three human readers and the ELISPOT reader. The Y axis contains the number of plaques identified in each instance, while the x axis shows the range of serial dilution from a starting titre of 50 pfu.

To assess the level of agreement between counts obtained by the ELISPOT reader and manual counting, Spearman correlation analysis was carried out. For this analysis, the mean plaque count at different dilutions of virus was obtained. The strength of association between the mean manual and ELISPOT counts measured using Spearman correlation analysis, was found to be high ($r=0.98$, $p<0.0001$), suggesting a high level of agreement between the manual and automated plaque counting techniques. The relationship between manual and automated plaque counting is shown graphically in figure 4.4. As a result of the high degree of agreement, all subsequent enumeration of plaques was carried out using the ELISPOT reader.

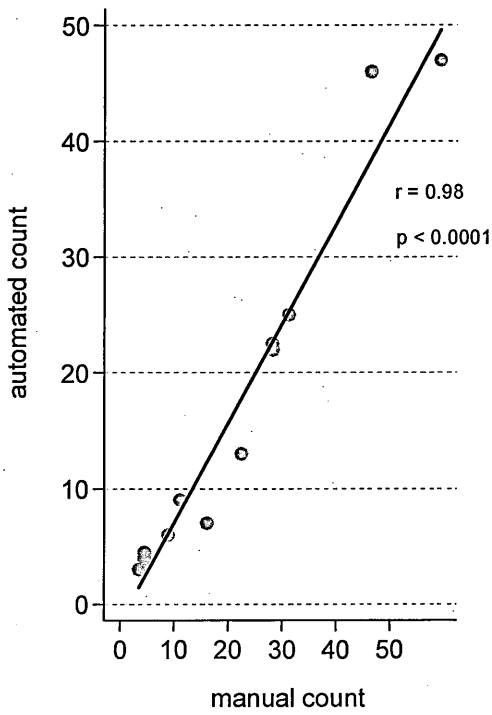


Figure 4.4 The relationship between automated and manual plaque counting is shown in this figure. The y axis represents the mean plaque count obtained by the ELISPOT reader, while the x axis represents mean counts by 3 manual readers. The association between the two methods was tested using Spearman's correlation analysis. The spearman correlation coefficient (r) and its corresponding p value are shown.

Calculation of neutralising dose 50 (ND_{50}) endpoint using the Spearman-Karber method

Neutralizing antibody titres in this study were measured as ND_{50} endpoint titres using the Spearman-Karber method (Cohen *et al.*, 2007). This method approximates the serum dilution required to halve the initial titre of virus, which in the case of this study, was approximately 50 plaques. Thus the neutralizing titre was estimated as the dilution of sera that permitted the development of approximately 25 plaques.

The Spearman-Karber formula is expressed as follows:

$\log_{10} ND_{50} = m - \Delta (\Sigma p - 0.5)$

Where m is the $\log_{10}$ dilution of the highest dilution of serum

(i.e. $\log_{10}$ (1/10,240) = -4.01)

and Δ is the constant interval between dilutions expressed as $\log_{10}$

(i.e. $\log_{10}$ (2) = 0.3010)

and $\Sigma p = x1/y + x2/y + x3/y + x4/y + x5/y + x6/y$

where x1 is the number of plaques for the first well, x2 for the second well and so on, and y is the mean number of plaques for the virus (no-serum) control wells.

Figure 4.5 shows an example of the neutralisation profile of strain A2 by different dilutions of infant serum.

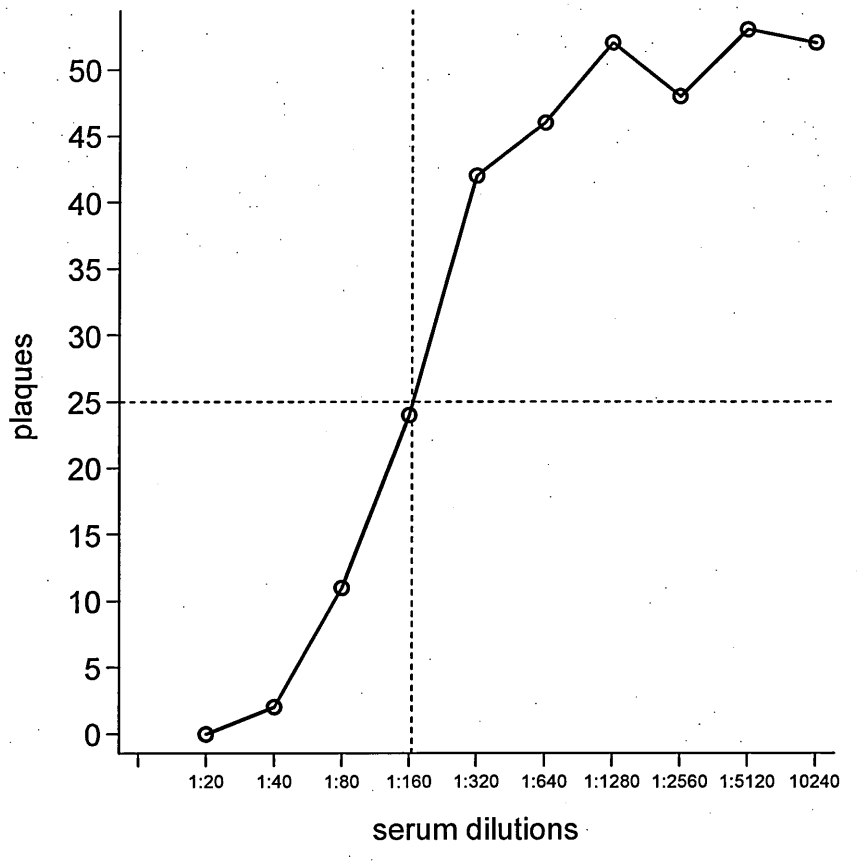


Figure 4.5 The neutralisation profile of RSV A2 by increasing dilutions of sera obtained from one infant is shown. The y axis shows the mean number of plaques that were observed at varying dilutions of serum (the serum dilutions are shown on the x axis). The dotted lines are used to indicate the serum dilution that results in the development of 25 plaques.

An example calculation of the neutralizing antibody titre as an ND_{50} endpoint titre using the Spearman-Karber method is shown below. Table 4.2 contains the mean plaque count at increasing dilutions of serum – these data are used in the titre calculation example.

Well number	1	2	3	4	5	6	7	8	9	10
mean plaque count	0	2	11	24	42	46	52	48	53	52
Serum dilution (1:x)	20	40	80	160	320	640	1280	2560	5120	10240

Table 4.2 The mean (of two) plaque counts at varying dilutions of infant sera are shown. These data are used in the example of titre calculation using the Spearman-Karber method

Implementation of the Spearman-Karber formula is shown in expressions *i-iv* below.

$Log_{10}\ ND_{50} = -4.01 - (-0.301\ (0/50+2/50+11/50+24/50+42/50+46/50+52/50+48/50$

$+53/50+52/50 - 0.5))$ ..... *i*

$Log_{10}\ ND_{50} = -2.17$ ..... *ii*

Anti-log of $-2.17 = 148$ ..... *iii*

ND_{50} (PRNT)= **148** ..... *iv*

For comparison, the neutralizing titre is inferred using the data shown on figure 4.5. This is done by using the linear part of the curve to estimate the serum dilution that corresponds to a plaque count of 25 on the y axis (shown using the dashed lines on figure 7). The ND_{50} value obtained through the Spearman-Karber formula is *148*, while that obtained by graphical inference is approximately *160*.

Chapter 5 - Neutralising antibody responses to natural RSV infection

Introduction

The group replacement dynamics of RSV at the population level (Cane *et al.*, 1994, Waris, 1991) suggest that population level immune selection is a key factor in the transmission of the virus. Mathematical modelling studies have shown that the assumption of group-specific immunity is sufficient to reproduce the observed group replacement dynamics (White *et al.*, 2005).

Despite RSV's ability to re-infect throughout life (Henderson *et al.*, 1979), individuals develop natural resistance against severe disease. While factors such as physiological maturation of the respiratory system may account for some resistance from severe disease (Dunnill, 1962), neutralising antibodies are thought to be a key feature of protective immunity and correlate strongly with protection from severe disease (Glezen *et al.*, 1981a) and infection (Hall *et al.*, 1991, Lee *et al.*, 2004). The role of antigenic variation in re-infection has to date not been clearly elucidated, however the extent of nucleotide and amino acid variation in the F and G genes of RSV A and B, provides some basis to suggest that protective immune responses targeted at these proteins may be variant specific. Although animal challenge studies have confirmed that the neutralising response is strongly group-specific, no conclusive data on the group-specificity of the human neutralising response has been published.

The failure by previous human cross neutralisation studies to reveal a group-specific component to the neutralising response (Coates *et al.*, 1963, Wulff *et al.*, 1964) may be attributed to certain study design shortcomings that may have obscured the ability to detect a significant difference between homologous and heterologous neutralising responses. The lack of definitive analysis of the molecular and antigenic characteristics of the infecting strains for example impedes proper interpretation of the data arising from these studies.

A number of studies have explored targets of the neutralising response. By use of monoclonal antibodies, a number of sites on the F and G proteins have been identified to be the targets of the neutralising response (Arbiza *et al.*, 1992, Lopez *et al.*, 1990, West *et al.*, 1994, Connor *et al.*, 2001, Agenbach *et al.*, 2005). Neutralising antibody epitopes have been identified in a number of sites on the F1 subunit of the F protein (Trudel *et al.*, 1987b, Lopez *et al.*, 1990, Martin-Gallardo *et al.*, 1991) as well as the central region of the G protein (Murata *et al.*, 2010, Garcia-Barreno *et al.*, 1992). Analysis of the location of these neutralising epitopes on the F proteins of wild-type strains of RSV has revealed that these epitopes are located on regions of the protein that contain a number of group conserved amino acids (Agenbach *et al.*, 2005). It is possible that amino acids that are conserved within but not between groups may have an impact on the specificity of the neutralising response directed at these epitopes.

The effect of pre-existing antibodies on the infant neutralising antibody response has been studied extensively. There is evidence that suggests that the infant neutralising response to infection is masked by maternally-derived antibodies (Murphy *et al.*, 1986b, Parrott *et al.*, 1973). For this reason, it is thought that vaccination in early

infancy is unlikely to be beneficial since the infant's native response to vaccination would be reduced in the presence of maternal antibody (Murata, 2009). It is however possible that the failure of young infants to mount a high neutralising response following infection or vaccination may be related to immunological immaturity and not solely the presence of pre-existing antibody of maternal origin. There is a paucity of data demonstrating the relationship between the ability to seroconvert to vaccination or natural infection and age among infants with a diverse range of pre-existing antibody titres.

Chapter Aims

This chapter aims to give a general description of the serum neutralising antibody response following natural infection in infants. It will explore the natural distribution of both acute and convalescent phase neutralising antibodies among paediatric RSV admissions. The relationship between acute stage neutralising antibodies and several clinical features of severe pneumonia will be explored with a view to identifying protective associations. It will further look at the effect of both age and pre-existing antibody on the ability of infants to seroconvert following natural infection. The genetic relationship between RSV A and RSV B infecting strains in terms of amino acid identity at both the F and G gene level will be explored. Regions on the F and G proteins of the infecting strains that are thought to be targets of neutralising antibodies identified from the published literature will be mapped on F and G protein sequences of infecting strains. This chapter will finally look at the group-specificity of the serum neutralising response following natural infection.

Methods

Study population, sampling and molecular characterisation of test and infecting viruses

Nasal washing samples were obtained from children less than 60 months of age admitted to Kilifi District Hospital (KDH) with syndromically defined severe or very severe pneumonia. RSV was diagnosed by Immunofluorescent Antibody Test (IFAT, Millipore Corporation). Multiplex RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) was used to determine if the infecting virus was from group A or B. An acute serum sample was collected from all children at admission and a convalescent serum sample from RSV positives approximately 4 weeks later. Nasal wash and serum samples were all stored at -80°C prior to use in the present study. Representative contemporary and historical strains of RSV were used as the test strains in this study. These were: the A2 genotype (RSV A; isolated in Australia in 1961), Kil/A/2006 (RSV A; Kenya, 2006), 8/60 (RSV B; Sweden, 1960) and Kil/B/2008 (RSV B; Kenya, 2008). The attachment and fusion protein genes of all the test viruses as well as a number of infecting strains were sequenced using previously described methods described in details in Chapter 3.

Plaque assay and microplaque reduction and neutralisation assay.

Titres of test strains were determined by plaque assay. Briefly, tenfold dilutions of test virus were made in minimum essential media (MEM) and inoculated onto HEp-2 cultures for 48 hours in 96-well plates. Cells were then fixed in methanol, washed and incubated at room temperature with a primary mouse anti-RSV IgG monoclonal antibody (Leica microsystems) followed by a secondary horseradish peroxidase linked

rabbit anti mouse IgG (Dako, Denmark). Plaques were developed using aminoethylcarbazole (AEC). An ELISPOT reader was used to count the number of plaques in each well. The plaque reduction neutralisation assay was carried out by preparing serial 2-fold dilutions of sera in MEM. 50 pfu of test virus were added to each dilution and following incubation for 1 hour at 4°C, the neutralisation reaction was inoculated onto HEp-2 cells and incubated at 37°C for 48 hours. Plaque development and enumeration were done as above. Neutralising antibody titres were calculated as neutralising dose 50 (ND_{50}) values using the Spearman-Karber method (Cohen *et al.*, 2007) and expressed as Plaque Reduction Neutralisation Titres (PRNT). These titres were normalised using $\log_{10}$ transformation for statistical analyses. A seroconversion was defined as a four-fold or greater rise in the neutralising antibody titre between the acute and convalescent phases of infection.

Statistical Analyses

Data analyses were done using Stata (version 11.1; StataCorp). Multiple regression analysis was used to compare the acute phase neutralising titres in different age classes. Comparison of homologous to heterologous responses in different age classes was done by comparing their mean fold rises in titre. Multiple regression analysis was used to test if the difference in the magnitude of the neutralising response to homologous virus (in terms of log fold rise in titre) in a particular age class was significantly different from the magnitude of the heterologous neutralising response in the same age class. Spearman rank correlation analysis was used to test the association between age and the magnitude of the neutralising response as well as the relationship between the acute phase response and the magnitude of the neutralising response. A logistic regression model was used to calculate the odds ratios of developing different features of severe

pneumonia in infants and children who had high or low tires of acute stage neutralising antibody. Finally, paired comparison of infant response to both homologous and heterologous virus was done using McNemar's $\chi 2$ test. The analytical output for the data shown in this chapter is presented in appendix 1.

Results

Age distribution and description of study participants

Neutralising antibodies were measured in the acute or convalescent sera of 118 paediatric pneumonia admissions with a median age of 4.7 months (range 0.2 – 41 months). 58.5% of the infants in the study were under 6 months of age, 21.2% were between 6 and 11.9 months of age while 20.3% were over 12 months of age. The age distribution of study participants is presented in figure 5.1. Most of the study participants were admitted with severe pneumonia. Very severe pneumonia was restricted to infants below 12 months of age while mild disease occurred only among infants who were over 12 months age (Table 5.1)

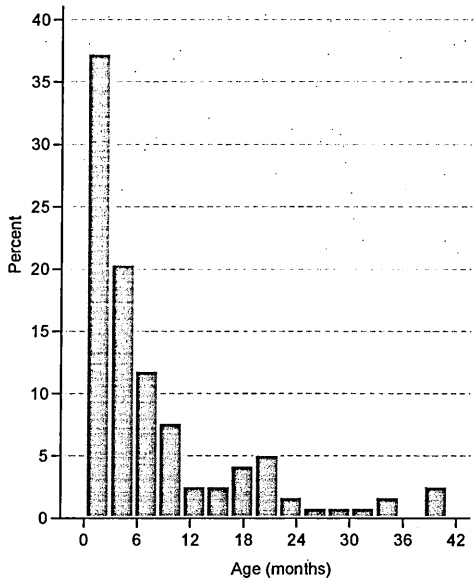


Figure 5.1 Distribution of ages at admission for paediatric inpatients whose acute and convalescent sera were used in this study

	WHO Pneumonia status (%)			
Age (months)	None	Mild	Severe	very severe
0 – 5	0	0	82.8	17.2
6 – 11.9	0	0	87.0	13.0
12+	13.0	13.0	74.0	0
Total	**2.73**	**2.73**	**81.82**	**12.73**

Table 5.1 The pneumonia status of infants and children admitted. The data are stratified by age.

The study participants were recruited in the course of 3 RSV epidemics: 20 individuals were recruited in the 2002/2003 RSV epidemic in Kilifi, 74 individuals were recruited from the 2005/2006 epidemic and 24 individuals were recruited from the 2007/2008 epidemic. During the 2002/2003 RSV epidemic, RSV A and B appeared to co-circulate in roughly equal proportions (Nokes *et al.* in preparation), while the 2005/2006 and 2008/2009 epidemics appeared to be strongly dominated by RSV A and B respectively.

RSV inpatient surveillance data in Kilifi District Hospital over the sampling period is shown in figure 5.2.

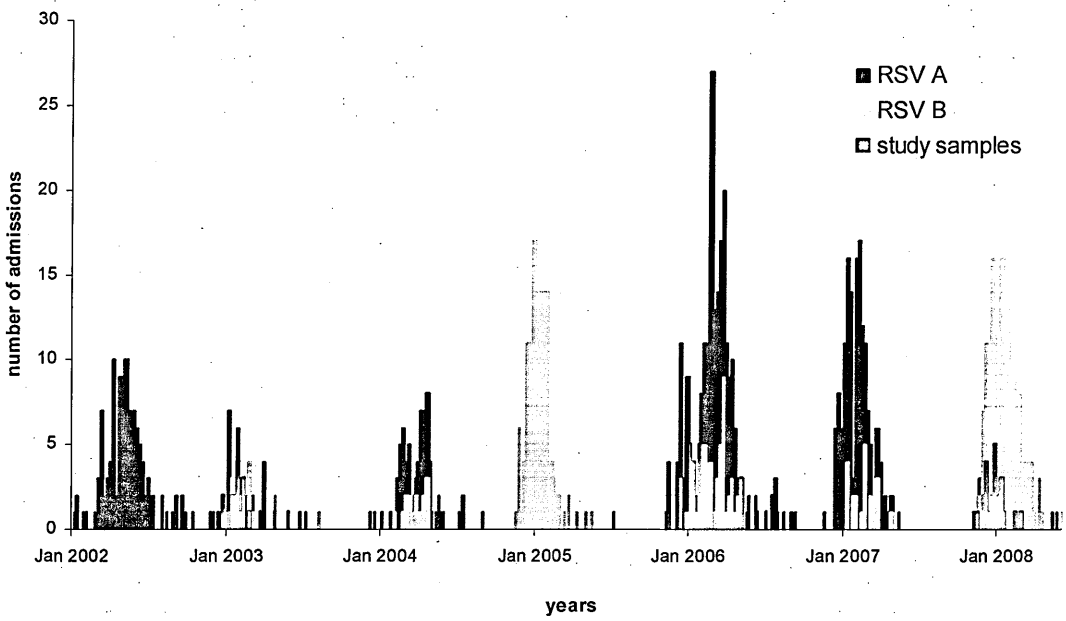


Figure 5.2 The temporal distribution of RSV A and B transmission over 7 years in Kilifi, Kenya. The dark bars represent transmission of RSV A, the grey bars represent transmission of RSV B while the red bars represent the samples that were used in this study. The temporal group replacement dynamics of RSV in Kilifi are also shown in figure 2.2

The infecting viruses isolated from each infant were classified as belonging to antigenic group A or B by multiplex PCR (Scott *et al.*, 2004, Stockton, 1998). 20 (100%) of the infants recruited in the 2002/2003 epidemic were infected with RSV B viruses that did not contain the 60 nucleotide duplication in the G gene (non-BA strains), 73 (98.6%) infants in the 2005/2006 epidemic were infected with RSV A while 1 (1.4%) was infected with RSV B, while 24 (100%) of the infants recruited in the 2008 epidemic were infected with RSV B viruses that contained the 60 nucleotide duplication. These data as well as the median ages of the study participants infected with either group are presented in table 5.2.

	Infecting group		
	A	B	
Epidemic	Median age months (n)	Median age months (n)	**Total**
2002/2003	N/A	7.1 (20)	7.1 (20)
2005/2006	4.06 (73)	8.2 (1)	4.1 (74)
2007/2008	N/A	6.2 (24)	6.2 (24)
Total	**4.6 (73)**	**7.1 (45)**	**4.7 (118)**

Table 5.2 The median ages of RSV A and B infected infants recruited into the study. The data are stratified by the epidemic during which the infections occurred.

Distribution of serum neutralising antibodies

Frequency distribution plots of neutralising antibodies at the acute and convalescent phases of infection were generated. The titres used in these analyses were mean titres obtained from four test viruses i.e. A2, 8/60, Ken/A/2006 and Ken/B/2008. The frequency distributions of the acute and convalescent phase neutralising antibody titres showed that both were skewed sharply to the left with median values of 216 PRNT (range 27 – 1309 PRNT) and 490 PRNT (range 47 – 1738 PRNT) respectively and corresponding mean values of 277 PRNT (SD 213) and 591 PRNT (SD 418) respectively (figure 5.3). Since many of the statistical analyses that were to be applied on these datasets are based on the presumption of normality, it was necessary to transform the data in order to normalise them. A logarithm 10 transformation was applied to both acute and convalescent antibody titres and the resulting distributions analysed for evidence of normality. The transformed distributions appeared to approach normality, with mean and median values generally occurring at the centre of the

distributions (figure 5.3). The log transformed acute and convalescent phase neutralising antibody distributions had medians of 2.3 PRNT (range 1.4 – 3.1 PRNT) and 2.7 PRNT (1.7 – 3.2 PRNT) respectively and means of 2.3 (SD 0.33) and 2.6 (SD 0.35) respectively. Since the log transformed data appeared to be normally distributed, they were used as the basis of all subsequent statistical analyses.

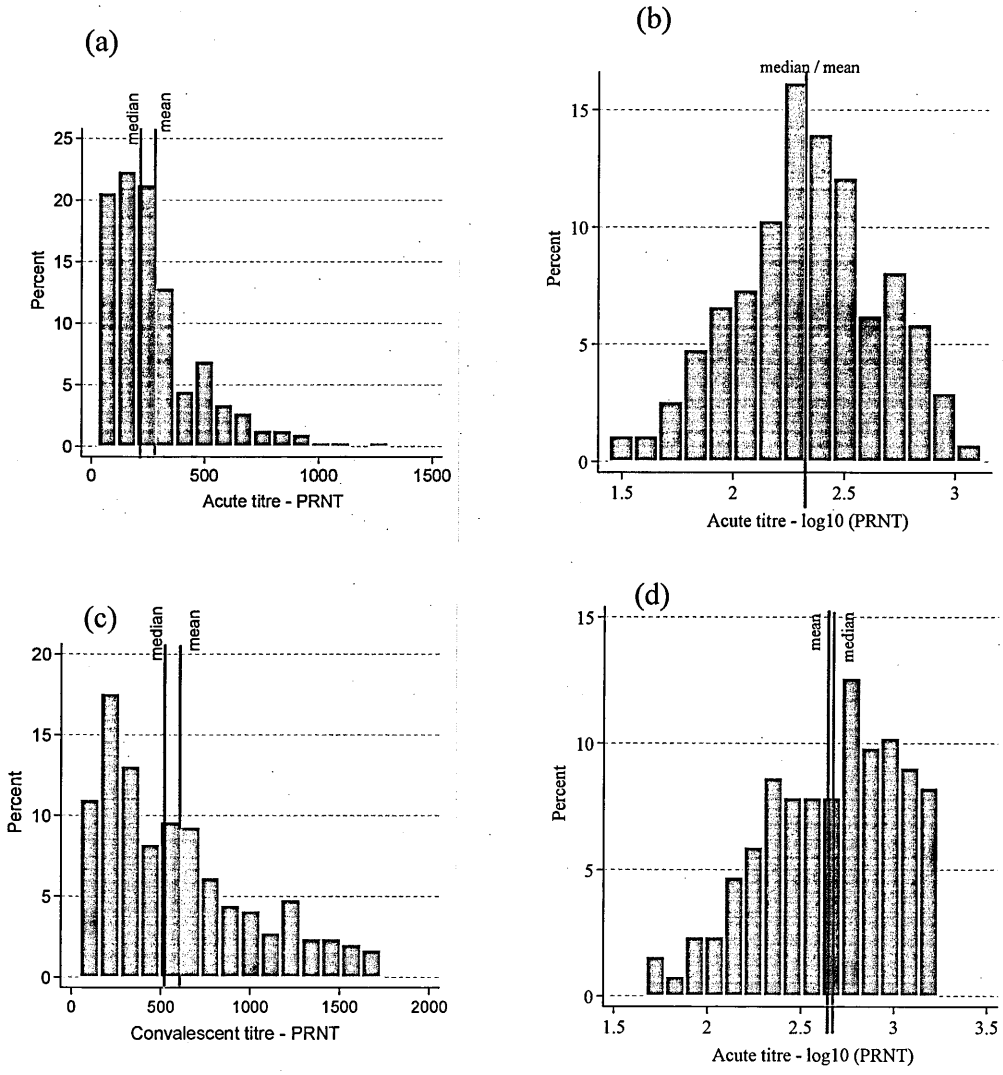


Figure 5.3 Acute and convalescent sera from 118 infants infected with RSV were normalised by logarithmic transformation. (a) Acute phase neutralising antibody titres expressed as Plaque Reduction Neutralisation Titres (PRNT) (b) Log transformed Acute phase titres expressed as log_{10} (PRNT) (c) Convalescent phase neutralising antibody titres expressed PRNT (d) Log transformed convalescent phase titres expressed as log_{10} (PRNT). The titres used in these analyses were mean titres to four test viruses (A2, 8/60, Ken/A/2006 and Ken/B/2008)

The age dynamics of serum acute and convalescent phase neutralising antibody responses of infants and children admitted with pneumonia

Distribution of acute and convalescent phase responses by age

In order to assess the magnitude of the neutralising response to natural infection an overlay of log transformed acute and convalescent titre distributions was generated (figure 5.4). Visual observation of the overlaid distributions showed marked separation between the acute and convalescent titres. There was a 2-fold difference between the mean acute antibody titre (2.3 $\log_{10}$ PRNT) and the mean convalescent antibody titre (2.6 $\log_{10}$ PRNT). The overlay of the acute and convalescent phase distributions is shown in figure 5.4.

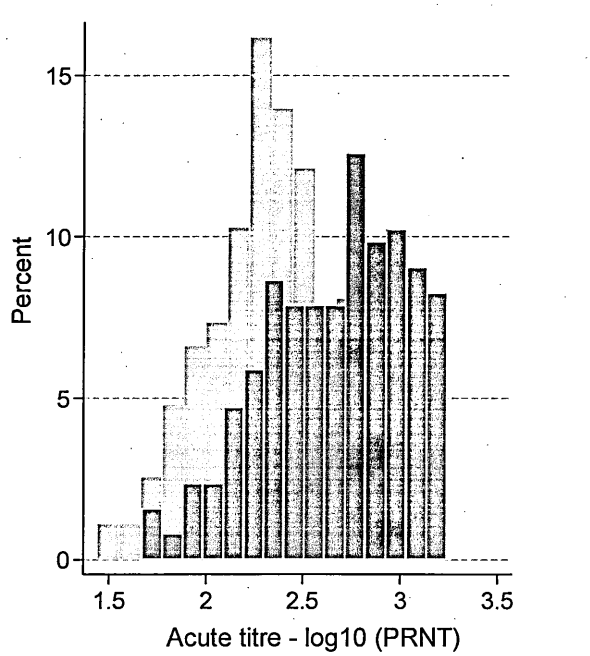


Figure 5.4 Overlay of log transformed acute and convalescent phase neutralising antibody titre distributions from the sera of 118 infants infected with RSV. Sera was tested against 4 test viruses (A2, 8/60, Ken/A/2006 and Ken/B/2008) and a mean titre obtained for use in these analyses. The light grey bars represent acute phase titres while the dark bars represent convalescent phase titres

In order to explore the effect of age on the neutralising response, the overlaid distributions were stratified by age. Three age classes were defined for these analyses: 0-5.9 months, 6–11.9 months and 12 months of age and above. The results of this stratification showed that age appeared to have a considerable influence on the neutralising antibody response to RSV. There was some difference between the acute and convalescent phase neutralising antibody distributions in the youngest age class (0-5.9 months of age) with mean values of 2.46 $\log_{10}$ PRNT and 2.54 $\log_{10}$ PRNT respectively ($t=-1.9$, $p=0.03$). This difference increased substantially in the 6-11.9 month age class, where the convalescent phase distribution shifted sharply to the right with a mean of 2.8 $\log_{10}$ PRNT and the acute phase distribution shifted to the left with a mean of 2.1 $\log_{10}$ PRNT. This difference was highly significant ($t=-12.4$, $p<0.0001$). In children over 12 months of age, the convalescent phase distribution shifted further to the right with a mean of 2.8 $\log_{10}$ PRNT relative to the mean acute phase titre of 2.3 $\log_{10}$ PRNT. This difference was also highly significant ($t=-8.6$, $p<0.0001$). These data are graphically depicted in figure 5.5.

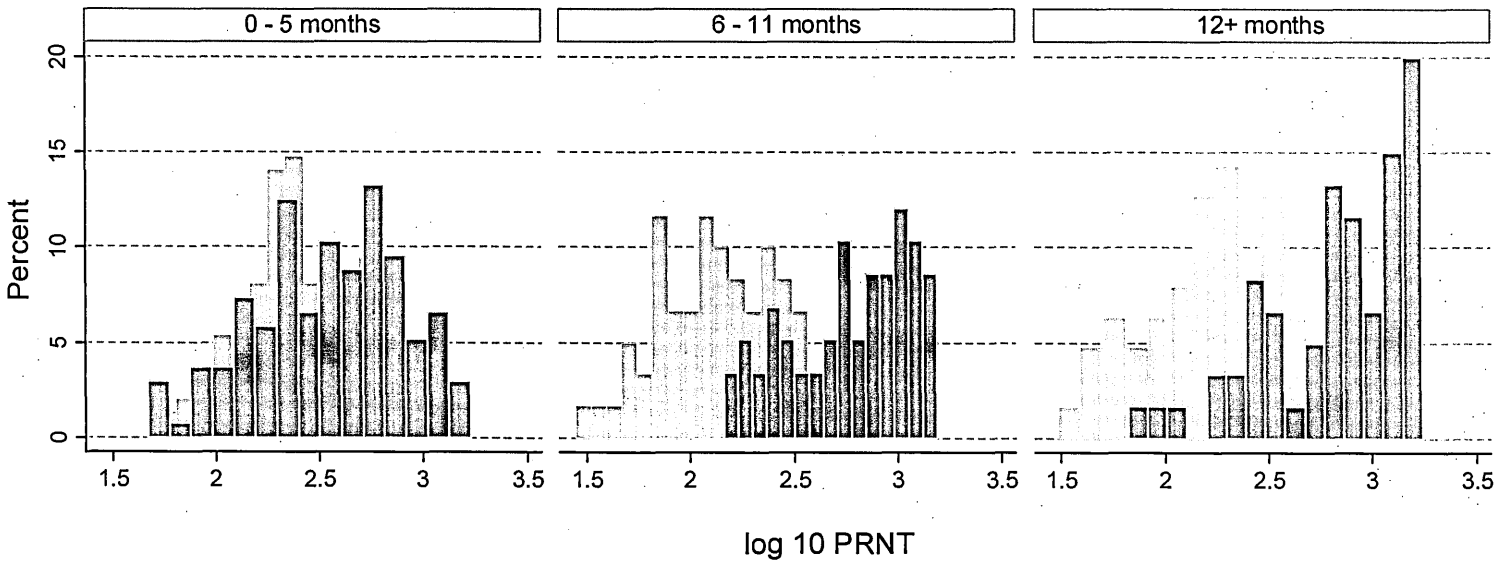


Figure 5.5 Age stratified comparison of acute and convalescent phase titre distributions Acute and convalescent phase distributions were overlayed in 3 age classes: 0-5months (n=69), 6-11months (n=25) and 12+ months (n=24). The mean titre from 4 test viruses was used in this analysis – i.e. A2, 8/60, Ken/A/2006 and Ken/B/2008.

Age dynamics of the acute phase response

The age dynamics of pre-existing antibodies were analysed in the acute phase samples obtained from infants of different ages. Neutralising titres were stratified into seven age classes: 0-0.9 (n=11), 1-1.9 (n=18), 2-2.9 (n=16), 3-3.9 (n=6), 4-4.9 (n=14), 5-5.9 (n=6), 6-11.9 (n=25) and 12+ (n=24) months of age. The neutralising titres used in these analyses were the mean titres to 4 test viruses: A2, 8/60, Ken/A/2006 and Ken/A/2008. The results of regression analysis showed that there was a progressive decline of acute phase antibodies in the first year of life. However comparison of the acute titres of infants in the 6-11.9 month age class with those of infants above 12 months of age showed that the older infants had a significantly greater mean titre of acute stage neutralising antibodies relative to the younger group ($p<0.0001$). These data are shown in figure 5.6.

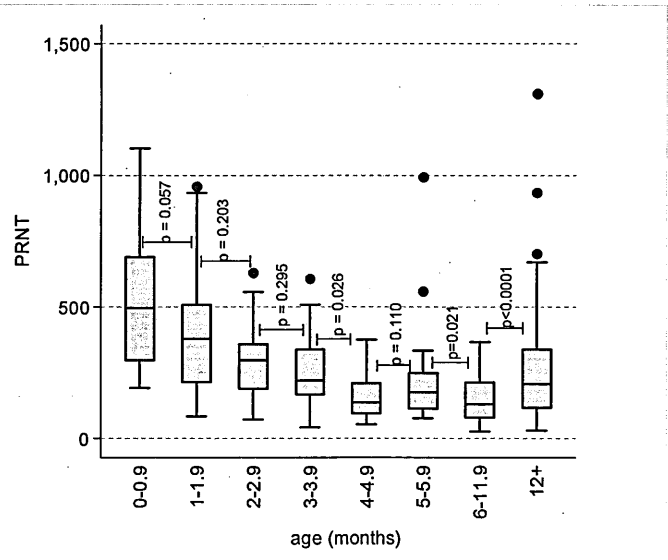


Figure 5.6 The dynamics of the acute phase response by age. Each bar represents the indicated age class. The difference in the mean neutralising titres successive age classes were tested for statistical significance (indicated by the p values). The lines in the middle of the boxes represent median titres while the lower and upper bounds of the boxes represent the 25th and 75th percentiles respectively. The upper and lower whiskers represent values that are up to 1.5 times below and above the 25th and 75th percentile respectively. The dots represent outliers which are values that fall outside the bounds defined by both the upper and lower whiskers.

Development of the neutralising response with age: comparison of the acute and convalescent responses.

The difference between the acute and convalescent phase responses was analysed by comparing the differences between these responses within the age structure defined above. The results of these analyses showed that in the youngest infants (0-0.9 months old) the mean convalescent phase response was significantly lower than the acute phase response (p=0.05). This difference was lost in the 1-1.9 (p=0.15), 2 -2.9 (p=0.09) and 3-3.9 (p=0.6) month age classes, where no difference between the acute and convalescent phase responses was seen. However the convalescent phase responses were significantly greater than the acute phase responses in the 5-5.9 (p=0.006), 6-11.9 (p<0.0001) and 12+ month age classes (p<0.0001). These data are shown graphically in figure 5.7.

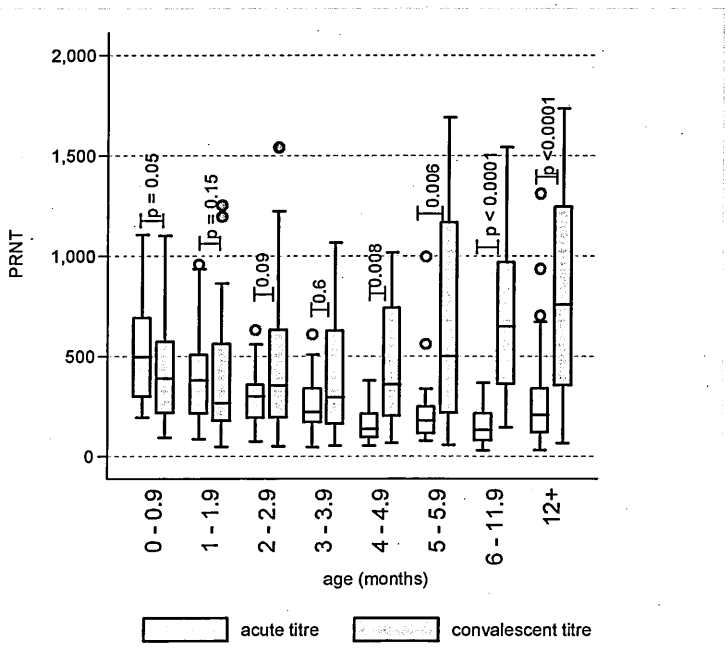


Figure 5.7 Comparison of the acute and convalescent neutralising response in different age classes. Description of the different elements of the box and whisker plots are as described in figure 5.6. The p values indicated on the line traversing the acute/convalescent distributions in a particular age class indicate whether the differences in mean titre are significantly different.

Relationship between seroconversion and age

The relationship between the ability to seroconvert following natural infection and age was analysed. A seroconversion was defined as a four-fold or greater rise in the convalescent phase response over the acute phase response. The proportions seroconverting in different age classes were calculated and presented graphically in figure 5.8. Analysis of seroconversion rates in different age classes showed an increasing trend with age. In general the seroconversion rate between 0 and 3.9 months of age was less than 20%. The seroconversion rate between 4 and 5.9 months of age was between 20% and 40% while the seroconversion rate among infants who were 6 months or older was above 40%.

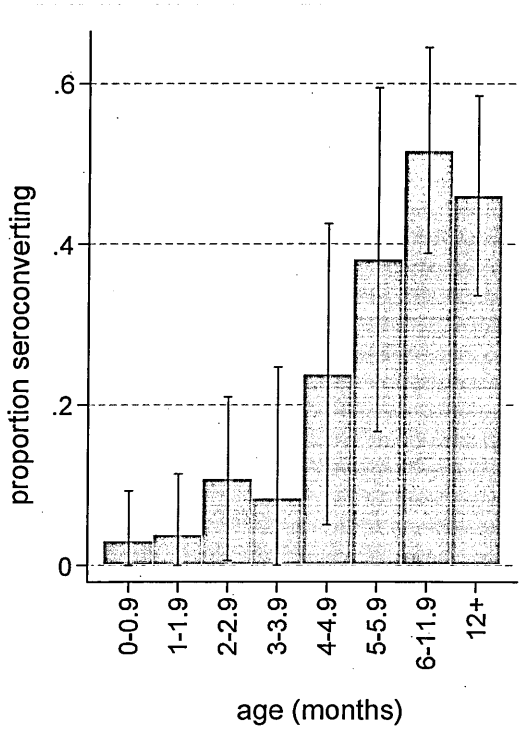


Figure 5.8 The proportions of infants seroconverting within different age classes A sero conversion was defined as a 4-fold or greater rise in titre from the acute to convalescent phases of infection. The height of the bars represent the proportion seroconverting within individual age classes while the lines traversing the bars indicate 95% confidence intervals about the proportions.

Next the relationship between the ability to mount a seroconversion and the titre of pre-existing antibodies was explored. The neutralising titres used in these analyses were the mean responses to 4 test viruses i.e. the A2, 8/60, Ken/A/2006 and Ken/B/2008. Acute stage neutralising titres were stratified into quartiles and the proportion of seroconversion in each quartile was calculated. There was a 90.2% seroconversion rate in the first quartile, a 59.6% seroconversion rate in the second quartile, a 13.5% seroconversion rate in the third quartile and no seroconversions (0%) in the fourth quartile (figure 5.9).

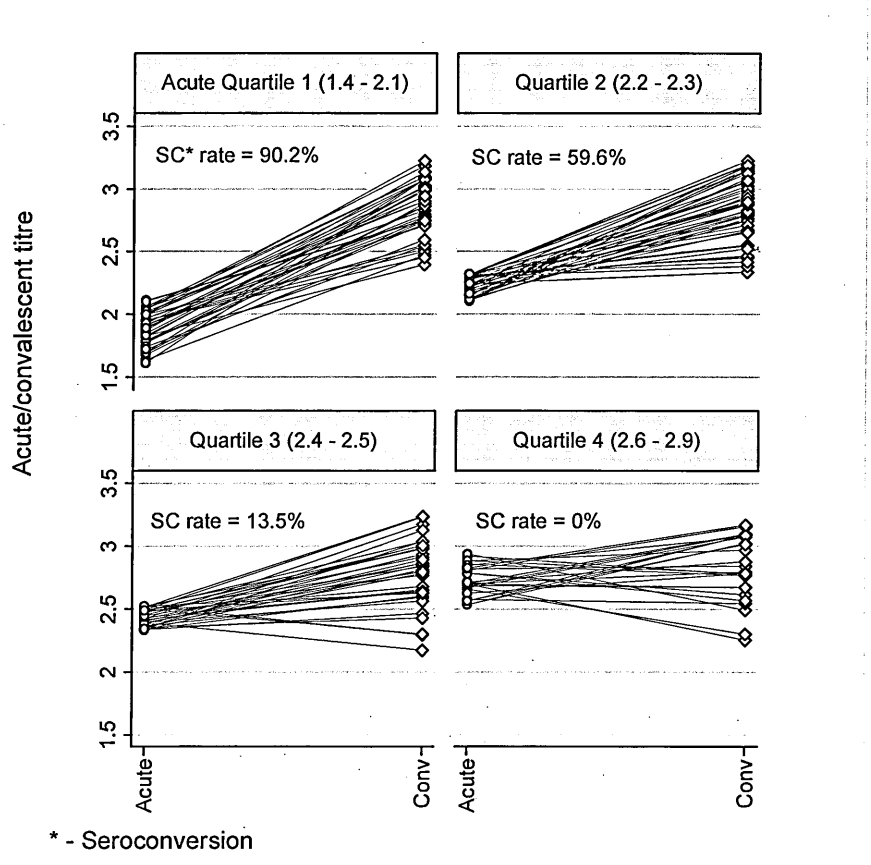


Figure 5.9 Acute phase titres stratified into quartiles and the proportions seroconverting in each quartile determined. The open circles denote the acute phase response, while the open diamonds denote the convalescent phase response

Spearman correlation analysis was used to test the strength of association between the magnitude of the neutralising response (in terms of fold rise in titre) and age on one hand and the titre of pre-existing antibody present in the acute phase sample on the other. The results of these analyses which are presented in figure 5.10, show that there was a strong and statistically significant negative association between pre-existing (acute stage) titres and the magnitude of the neutralising response ($r=-75$, $p<0.0001$) and a moderate but statistically significant positive association between the magnitude of the neutralising response and the age ($r=0.6$, $p<0.0001$).

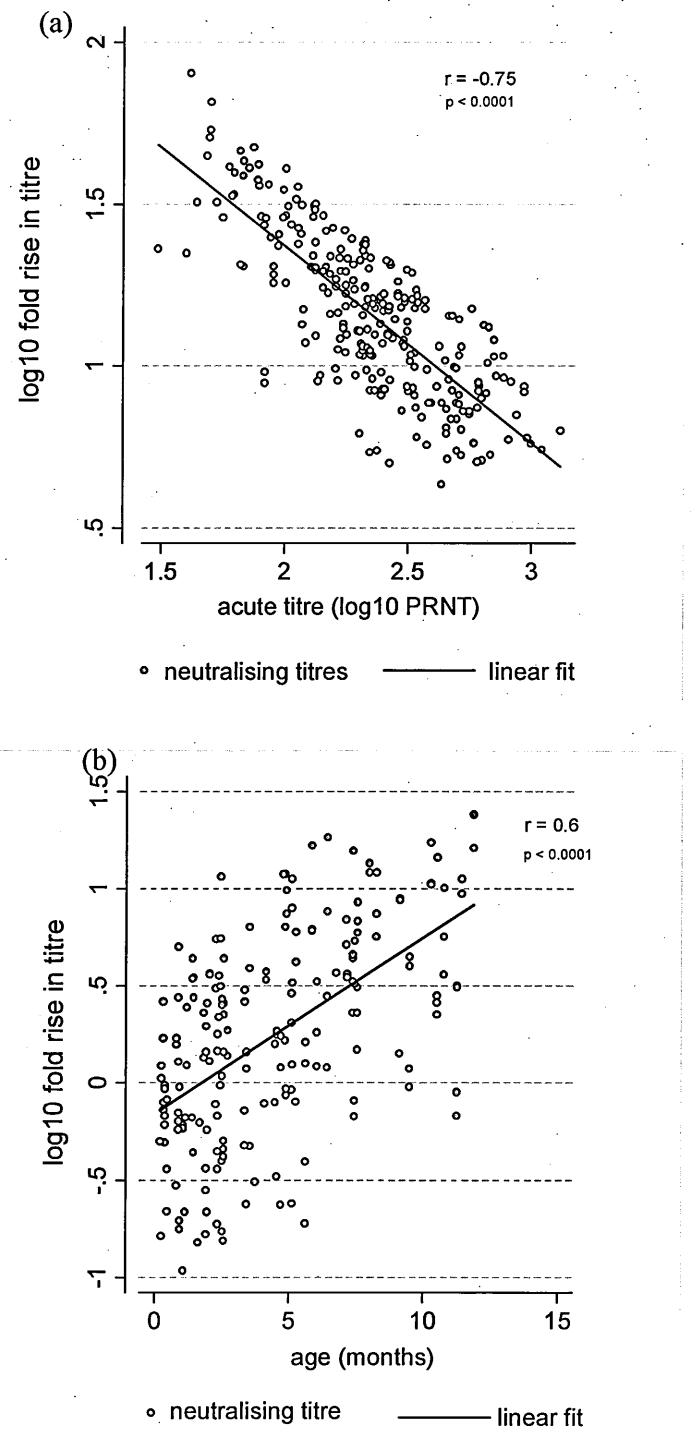


Figure 5.10 Relationship between the magnitude of the neutralising antibody response and (a) the acute phase titre and (b) age of infant. The y axes denote the magnitude of the change in neutralising antibody response expressed as $\log_{10}$ fold rise in titre from the acute to convalescent phase of infect. The x axes in (a) and (b) denote the acute phase titres and age of the infant respectively. Spearman correlation coefficients (r) and corresponding p values are indicated on each panel.

The relationship between neutralising antibodies and severe pneumonia

The relationship between the acute phase neutralising antibody titre and severity of RSV infection was explored by comparing the clinical outcome of disease and the neutralising titre at the acute stage of infection. The neutralising titres used in these analyses were the mean titres to 4 test viruses (A2, 8/60, Ken/A/2006 and Ken/B/2008). The acute sera of 106 infants for whom clinical pneumonia syndromic data were available were included in these analyses. Multivariate logistic regression analysis was used to estimate the relationship between acute phase neutralising antibodies and the following clinical features of WHO defined severe or very severe pneumonia: shock, flaring, indrawing, wheeze, hypoxia and crackles. A cutoff titre of $\log_{10}$ 2.5 PRNT was selected in order to categorise acute stage titres in two classes: high and low level titres. The value of $\log_{10}$ 2.5 PRNT was derived from a comparison of the frequency distributions of acute and convalescent phase antibodies since it appeared to be the point of separation between the two distributions (figure 5.4). The results of these analyses showed some evidence of protection by neutralising antibodies against shock (OR=0.3; p=0.016). Although not statistically significant, the odds ratios for flaring (OR=0.7; p=0.3), hypoxia (OR=0.7; p=0.6) and crackles (OR=0.8; p=0.6) were all below 1. There was no protective association between wheeze and indrawing and the acute stage neutralising response (figure 5.11).

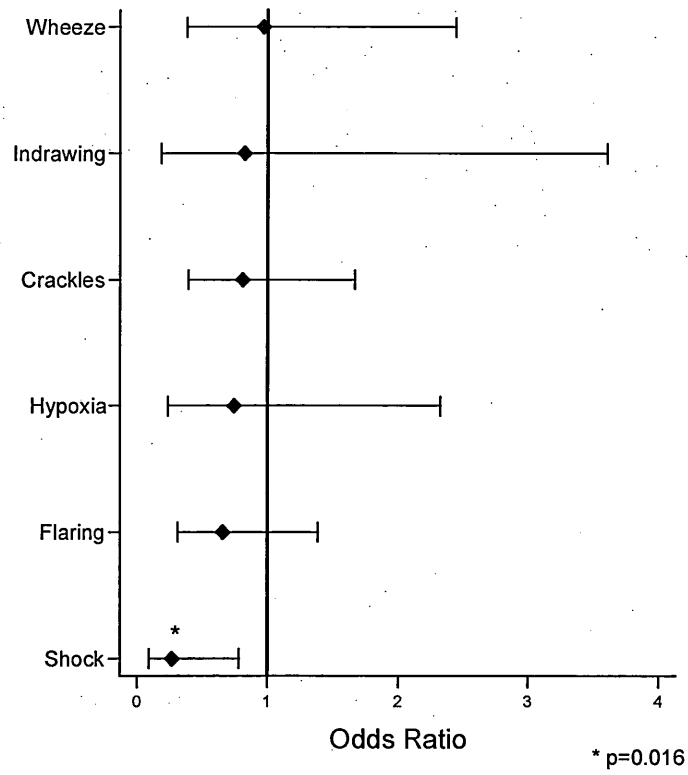


Figure 5.11 The Odds Ratios of developing various clinical features of severe pneumonia among infants with high (>=2.5 $\log_{10}$ PRNT) and low (<2.5 $\log_{10}$PRNT) acute phase neutralising antibody titres. A statistically significant association between high titres of pre-existing neutralising antibodies and protection from shock was found in the analysis.

Analysis of the genetic relationship between infecting RSV A and B strains

The F and G genes of 60 infecting RSV A and B viruses were partially sequenced in order to infer the level of genetic and antigenic relatedness between them. The region between nucleotide 284 of the G gene and nucleotide 9 of the F gene was sequenced. In the case of the F gene, the region between nucleotides 121 and 918 of the F gene was sequenced. Nucleotide and deduced amino acid sequences from different infecting strains were manually aligned and each position within the alignment analysed for evidence of variation. There was 78.7% nucleotide sequence identity between the F genes of infecting RSV A and B and 87.6% amino acid identity

between them. Infecting RSV A and B strains shared 45% nucleotide sequence identity at the G gene level and only 28.6% amino acid identity. These data are shown in table 5.3.

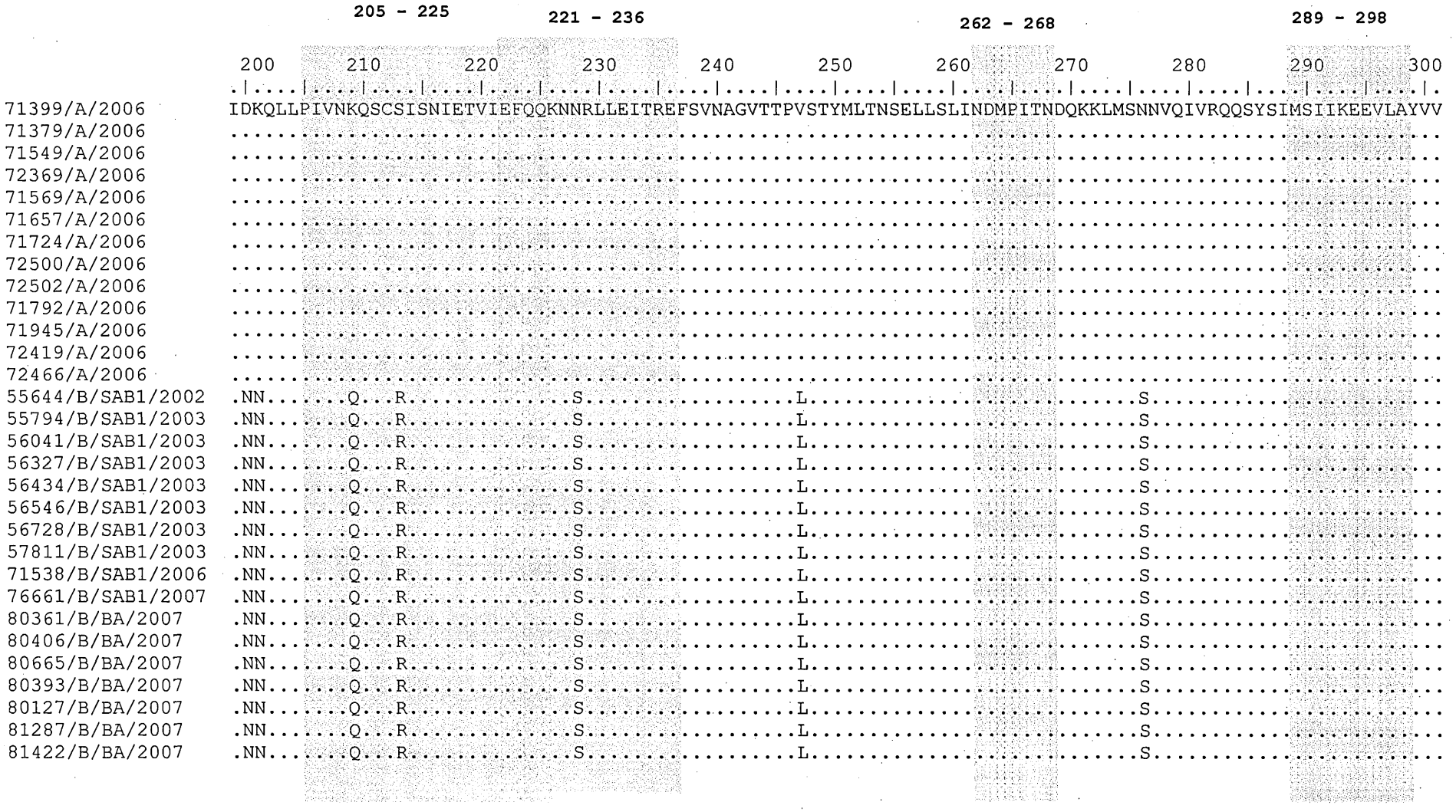
Protein/gene	% identity (RSV A and B)	
	Nucleotide	Amino acid
F	78.7	87.6
G	45.2	28.6

Table 5.3 The genetic relatedness between infecting strains of RSV A and B at the level of the F and G gene.

Analysis of variation on putative neutralising epitopes on the deduced F protein amino acid sequences of infecting strains.

Amino acid sequences of four neutralising antibody epitopes on the F protein were obtained from the published literature (Trudel *et al.*, 1987b, Lopez *et al.*, 1990, Martin-Gallardo *et al.*, 1991, Bourgeois *et al.*, 1991) and mapped on the F protein amino acid alignments of infecting strains. Each amino acid position was then analysed for evidence of conservation between and within groups. Two of the four neutralising antibody epitopes that were identified were located in regions of the protein that contained amino acids that were conserved within but not between groups. The first of these occurred between amino acid 205 and 225 (Bourgeois *et al.*, 1991) where the glutamine (Q) residue at position 209 was perfectly conserved in all RSV B isolates sequenced but occurred as a perfectly conserved lysine (K) residue

in all the RSV A patient isolates. The amino acid residue at position 213 within this epitope contained a serine (S) that was perfectly conserved in all RSV A isolates sequenced but that changed to isoleucine (R) in all RSV B isolates. Analysis of the neutralising antibody epitope that occurs between amino acids 221-236 (Trudel *et al.*, 1987b) showed that the amino acid at position 228 within this epitope occurred as a conserved asparagine (N) in all RSV A isolates but changed to a Serine (S) in all sequenced RSV B isolates. The last two neutralising antibody epitopes that were analysed for evidence of group-specific variation occurred between amino acid residues 262-268 (Lopez *et al.*, 1990) and 289-298 (Martin-Gallardo *et al.*, 1991) and were found to be located on regions of the F protein that were perfectly conserved within and between the two groups. Figure 5.12 graphically shows the position of these epitopes within the deduced F protein amino acid sequence alignments of infecting strains.



28Figure 5.12 Mapping of selected neutralising antibody epitopes on an alignment of a limited set of infecting F protein amino acid sequences.

Analysis of variation on putative neutralising epitopes on the deduced G protein amino acid sequences of infecting strains.

Analysis of neutralising antibody epitopes on the deduced G protein amino acid sequences of infecting strains was then carried out. Two neutralising antibody epitopes on the G protein were identified in the published literature (Garcia-Barreno *et al.*, 1992, Murata *et al.*, 2010) and highlighted on an alignment of deduced G protein amino acids of infecting strains. The first epitope was located between amino acids 151 and 172 of the G protein (Murata *et al.*, 2010) while the second was located between amino acids 201 and 213 (Garcia-Barreno *et al.*, 1992) – figure 5.13. Both epitopes were located on regions of the G protein that contained multiple group conserved amino acids as well as a smaller number of genotype-specific amino acids. The epitope located between amino acids 151-172 (Murata *et al.*, 2010) contained 7 amino acids that were conserved within but not between groups, meaning that 32% of the amino acid content on this epitope was group-specific. Notably, the terminal 9 amino acids of this epitope's 22 amino acids fell within the 13 amino acid motif that is perfectly conserved in all human RSV isolates (between amino acids 164 -176). At least one amino acid within this epitope was genotype-specific. The amino acid at position 160 contained an asparagine residue that was conserved in all the RSV A and RSV B isolates from the 2007/2008 epidemic (BA strains) but changed to a lysine in the RSV B isolates from the 2002/2003 epidemic (non-BA strains). Within the neutralising antibody epitope that fell between amino acids 201-213 (Garcia-Barreno *et al.*, 1992), 3 amino acids (23% of the total epitope amino acid content) were conserved within but not between groups. There was one genotype-specific amino acid within this epitope: the amino acid at position 207 contained a threonine residue that was conserved in all RSV A isolates and all RSV B isolates that did not contain

the 60 nucleotide duplication (non-BA) but which changed to either a leucine or proline in BA strains of RSV B. Figure 5.13 is a graphic depiction of the location of these epitopes on an alignment of deduced G protein amino acid sequences of infecting strains.

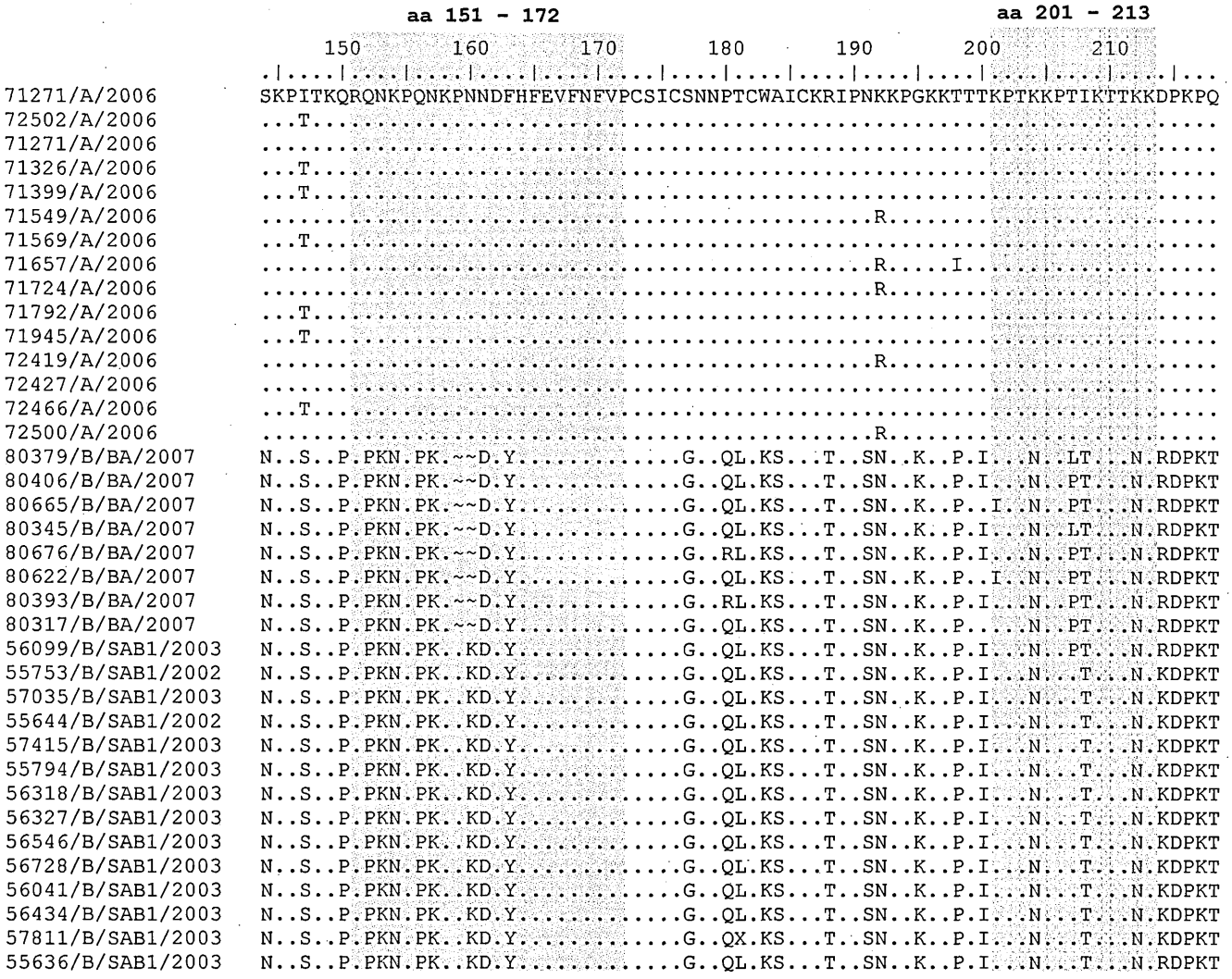


Figure 5.13 Mapping of selected neutralising antibody epitopes on an alignment of a limited set of infecting G protein amino acid sequences.

Group-specificity of the neutralising antibody response

Neutralising antibody titres were classified as homologous if the group designation of the infecting strain matched that of the test strain and heterologous if the test and infecting strains were discordant at group level. Analyses of the group-specificity of the neutralising response were stratified by age to account for age specific differences in the infant response to RSV. Individuals were considered to have made significant responses to a specific virus strain if they made a four-fold or greater rise in the neutralising antibody response.

Sera from 32 RSV A infected individuals were assayed for neutralising antibody against both Kil/A/2006 and Kil/B/2006. Of these, the proportion that seroconverted to genotype Kil/A/2006 (50%) was significantly greater than the proportion that seroconverted to genotype Kil/B/2008 (12.5%; McNemar's χ^2=12; p=0.0005). Similarly sera from 25 RSV B infected individuals were assayed for neutralising antibody against Kil/A/2006 and Kil/B/2006 and of these the proportion who seroconverted to genotype Kil/B/2008 (40%) was significantly greater than the proportion who seroconverted to genotype Kil/A/2006 (8%; McNemar's χ^2=8, p=0.008).

Of 18 RSV A infected individuals whose sera were tested for neutralising antibody against prototype viruses A2 and 8/60, the proportion of individuals who seroconverted to the A2 genotype (28%) was greater than the proportion who seroconverted to the 8/60 (0%) genotype. This difference was found to be borderline significant (McNemar's χ2=5, p=0.06). Among 20 RSV B infected individuals

whose sera was assayed for neutralising antibody against both the A2 and 8/60 strains, there was a statistically significant difference between the proportion that seroconverted to genotype 8/60 (65%) and the proportion that seroconverted to the A2 strain (10%; McNemar's$\chi 2=11$, $p=0.001$).

Comparison of the homologous and heterologous neutralising antibody responses in different age classes was also done by analysing the differences between homologous and heterologous fold rises in titre. The test viruses in these analyses were local RSV A and B strains Ken/A/2005 and Ken/B/2008. In each age class the fold rise in titre to both homologous and heterologous virus was calculated by dividing the convalescent phase titre with the acute phase titre and the resulting figures normalised using a log 10 transformation. Multiple regression analysis was used to compare homologous and heterologous rises in titre to both RSV A and B. The homologous response to RSV A by RSV A infected individuals was significantly greater than their heterologous response to RSV B in (a) the 0-5 month age class (1.8 fold rise in titre vs. 0.5 fold rise in titre; $p<0.0001$), (b) the 6-11 month age class (11.2 vs. 3.8; $p=0.002$) and (c) the 12+ month age class (7.1 vs. 2.2; $p=0.001$). Similarly the homologous response to RSV B by RSV B infected individuals was significantly greater than their heterologous response to RSV A in (a) the 0-5 month age class (2.7 vs. 0.7; $p<0.0001$), (b) the 6-11 month age class (5.9 vs. 1.7; $p<0.0001$) and (c) the 12+ month age class (4.3 vs. 0.9; $p<0.0001$). These data are graphically depicted in figure 5.14.

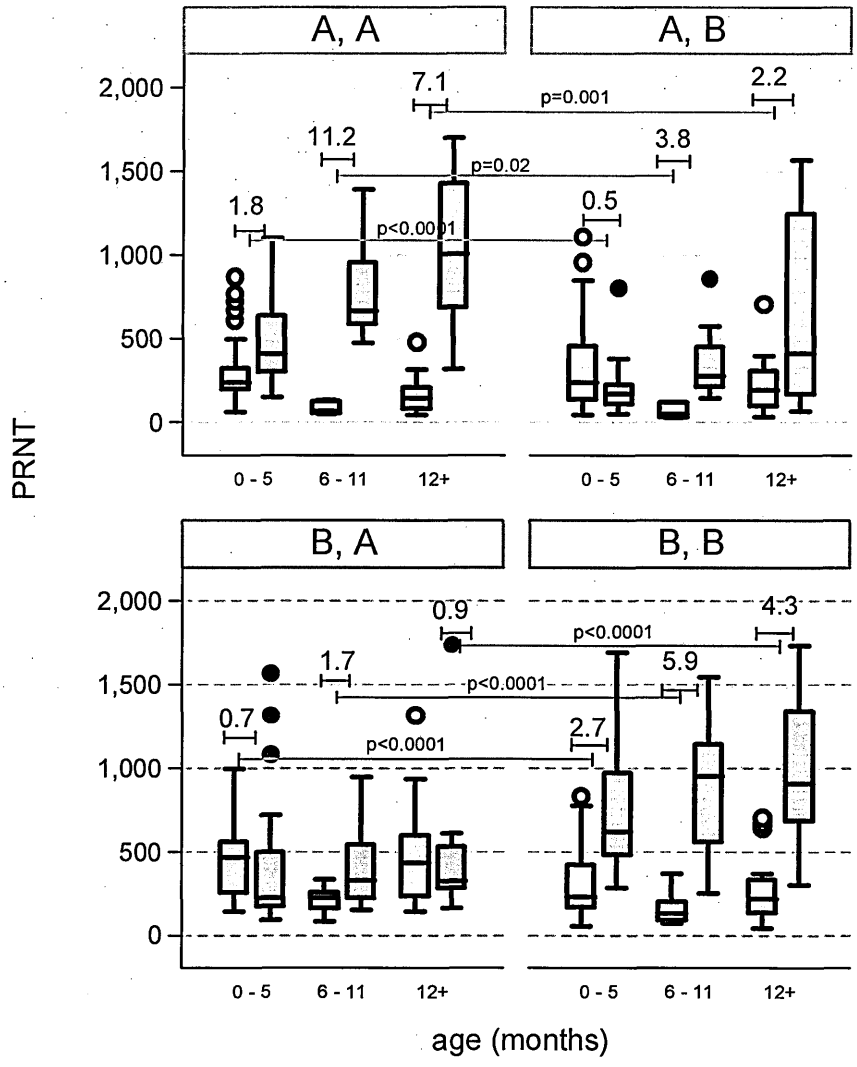


Figure 5.14 Group-specific neutralising antibody responses to RSV infection. Acute and convalescent neutralising antibody responses to homologous and heterologous virus were compared among 118 infants with natural RSV infection. The first letter in each panel heading denotes the group designation of the infecting virus while the second letter denotes the group designation of the test virus. The light grey bars indicate the distribution of the acute phase response while the dark grey bars denote the distribution of convalescent responses. The lines in the middle of the boxes represent median titres while the lower and upper bounds of the boxes represent the 25th and 75th percentiles respectively. The upper and lower whiskers represent values that are up to 1.5 times below and above the 25th and 75th percentile respectively. The dots represent outliers which are values that fall outside the bounds defined by both the upper and lower whiskers. The x axis represents the age classes that were analysed while the y axis represents the neutralising antibody titre (PRNT). The number above each acute/convalescent pair denotes the mean fold rise in titre from the acute to convalescent phases of infection. Comparison of the magnitude of response (in terms of fold rise in titre) to homologous virus and heterologous virus is shown by the long bars traversing the panels. The p value denotes whether the difference between the homologous and heterologous response in a particular age class is statistically significant.

To account for the possible confounding of primary responses by prior undetected infections, a 69 infants born during an inter-epidemic period and who experienced what was assumed to be their first infection in the epidemic immediately following their births was selected. The odds ratio of seroconverting to both homologous and heterologous virus between this group and everyone else in the study was carried out using multiple logistic regression analysis. The results of this analysis showed that there was no difference in the ability of infants within the two groups to seroconvert to homologous or heterologous virus (Odds Ratio 1.8, p=0.4). Comparison by multiple linear regression analysis of the neutralising response of individuals who were born prior to the start of an epidemic and those born within an epidemic showed that there was no difference in their responses to homologous or heterologous virus. The mean homologous response to RSV A was no different between individuals born prior to the start of an epidemic and those born during the epidemic (4-fold rise in titre vs. 3.1 fold rise in titre; p=0.4) nor was there a difference in their heterologous response to RSV B (1.1 vs. 0.7; p=0.3). Similarly, the homologous RSV B response by individuals born prior to an epidemic was no different from the response of individuals born in the course of an epidemic (4.2 vs. 3.7; p=0.4). There was similarly no difference in their heterologous response to RSV A (1.2 vs. 0.8; p= 0.3). These data are presented graphically in figure 5.15.

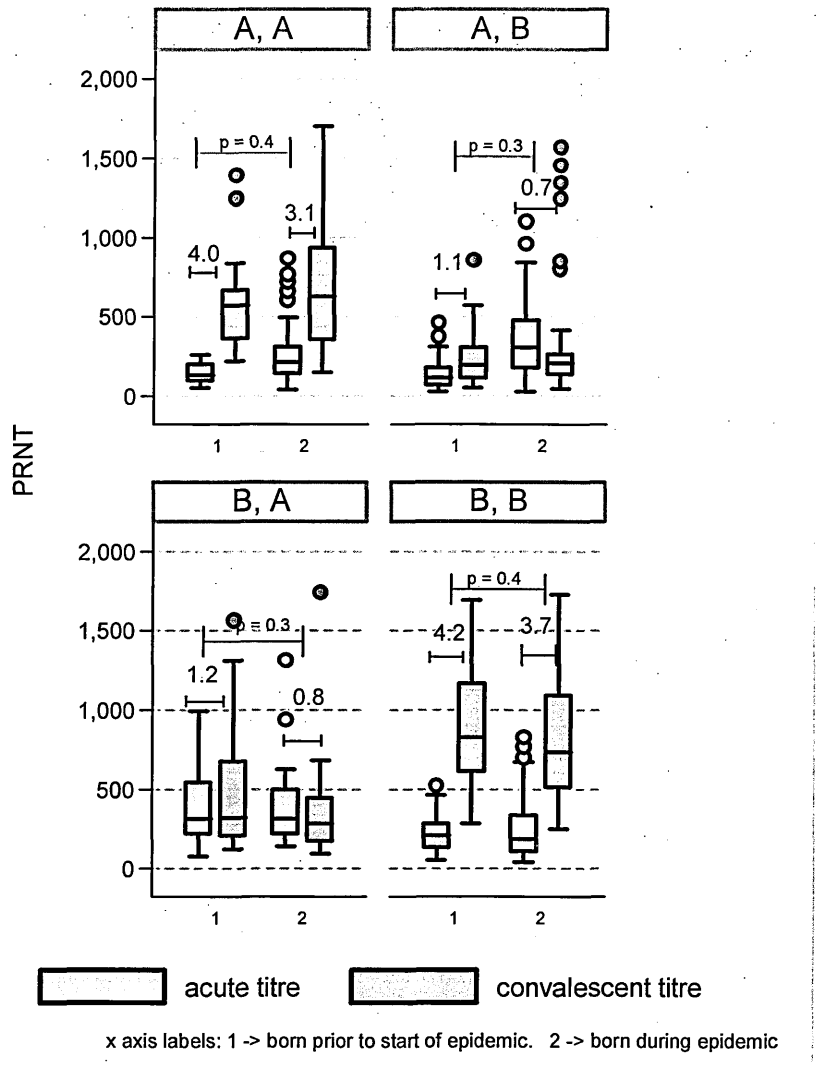


Figure 5.15 Comparison of neutralising homologous and heterologous neutralising responses among infants born at different times. The first letter in each panel heading denotes the group designation of the infecting virus while the second letter denotes the group designation of the test virus. The y axis shows the neutralising antibody titre (PRNT) while the x axis denotes the time of birth relative to the epidemic in which the infection occurred. The number above each acute/convalescent pair denotes the fold rise in titre. The p values denote whether the mean fold rise in titre to homologous or heterologous virus, varies by time of birth.

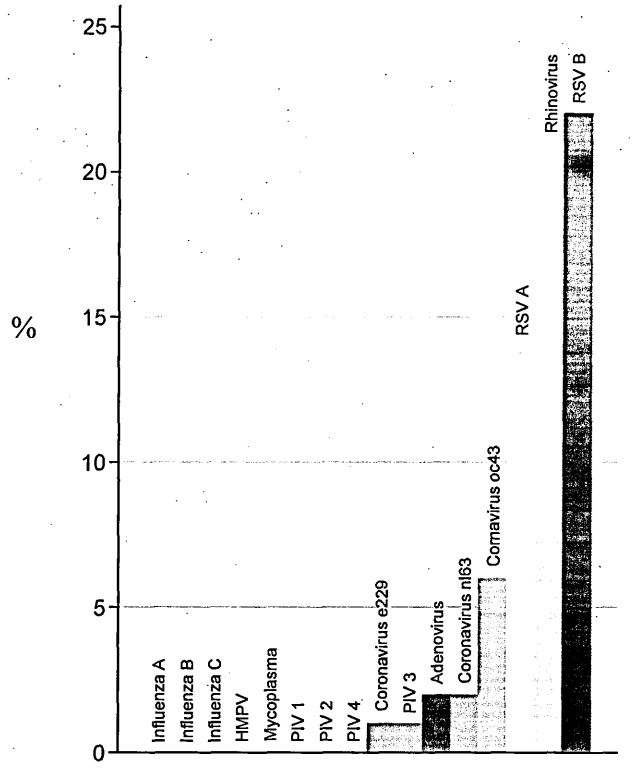


Figure 8.1 The total number of respiratory pathogens identified in the household study samples (n=100) analysed in this chapter.

Association between cytokine/chemokine concentration/viral load and URTI in infants

The effect of viral load (recorded as the reciprocal of cycle threshold multiplied by 10) on illness among infants infected with RSV was evaluated by comparing mean RSV viral load in samples collected in the presence of Upper Respiratory Tract Infection (URTI) to viral loads in samples collected in its absence. Samples collected in the presence of URTI had significantly greater viral titres compared to samples collected in the absence of URTI. Figure 8.2 shows the distribution of viral loads among these two groups.

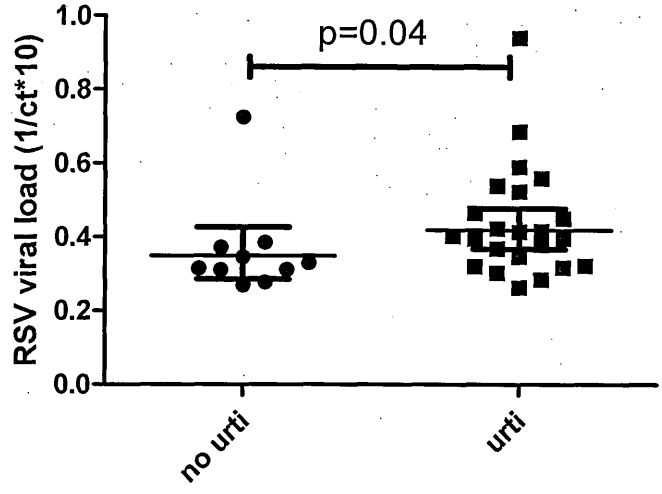


Figure 8.2 The relationship between RSV viral load and the presence (n=23) or absence (n=10) of URTI in infants who an RSV infection

The contribution of different cytokines/chemokines in the development of URTI among infants irrespective of the infecting pathogen was assessed by comparing cytokine/chemokine concentrations in nasal samples collected in the presence of URTI to concentrations in nasal samples collected in the absence of URTI. 58% (n=58) of the nasal samples collected in this study were collected during an URTI episode. Nasal secretions collected during URTI episodes had significantly greater concentrations of IL-1β, IL-6, IL-8, TNF-α, MCP-4, MDC, MIP-1 β, IFN-γ and IL-10 relative to those collected in the absence of URTI. These data are shown in figure 8.3.

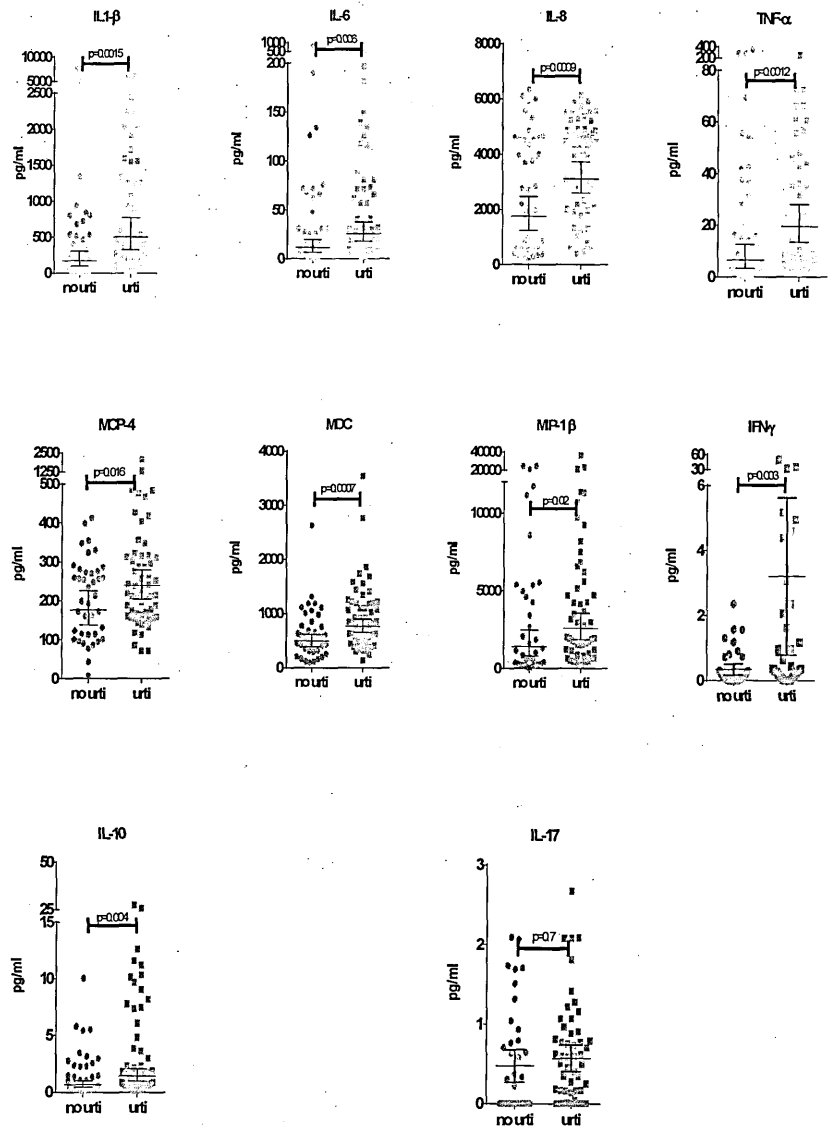


Figure 8.3 The distribution of different cytokines/chemokines in samples obtained from infants in whom symptoms of URTI were present (urti; n=58) or absent (no urti; n=42). Geometric mean concentrations and 95% confidence intervals are also indicated

Comparison of cytokine/chemokine responses in RSV positive and rhinovirus positive samples

Samples found to be positive for either RSV or rhinovirus and negative for all other respiratory pathogens were compared for evidence of differential cytokine/chemokine production. Out of 100 samples tested for 16 respiratory pathogens, RSV was present as the only respiratory pathogen detected in 29 samples (out of a total of 33 RSV positive samples) while rhinovirus was exclusively detected in 21 samples (out of 21 rhinovirus positive samples). The total number of RSV and rhinovirus positive samples, irrespective of co-infection status is presented in table 8.1.

RSV present	Rhinovirus present		Total
	No	Yes	
No	46	21	67
Yes	33	0	33
Total	79	21	100

Table 8.1 Distribution of samples in the household study which were found to be positive for RSV, rhinovirus, both viruses or none of the viruses

A comparison of cytokine/chemokine concentrations in nasal secretions in which either RSV or rhinovirus was detected as the only respiratory virus is presented in figure 8.4. In general the levels of most cytokines/chemokines were higher in the presence of rhinovirus relative to RSV. The mean concentrations of IL-1β, IL-8, TNF-α, MDC and MIP-1 β were significantly greater in the presence of rhinovirus compared to RSV. In contrast, the mean concentration of IL-17 was greater in the presence of RSV relative to rhinovirus, although this difference was just below statistical significance ($p=0.06$). The mean concentrations of IL-6, MCP-4, IFN-γ and IL-10 were no different in the presence of either RSV or rhinovirus.

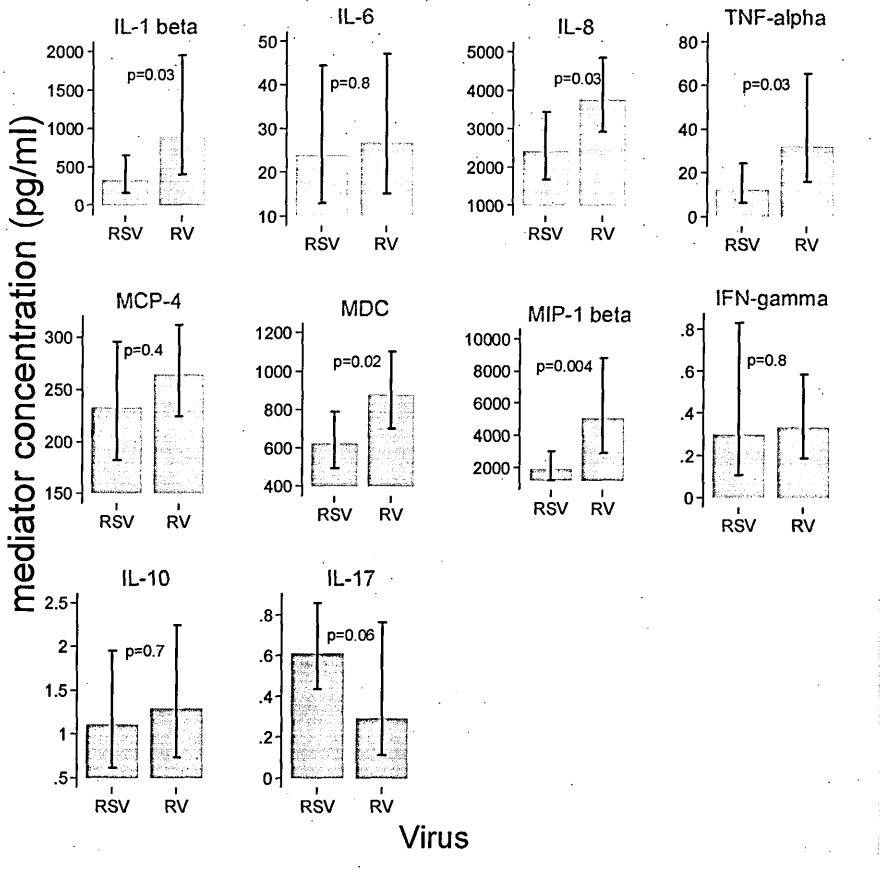


Figure 8.4 Comparison of geometric mean (with 95% CI) cytokine/chemokine concentrations in samples that were positive exclusively either for RSV (n=29) or rhinovirus (n=21)

Association between RSV Viral Load and cytokine/chemokine response

The association between RSV viral load and cytokine/chemokine concentration was measured using Spearman correlation analysis. In this study, an indirect measure of viral load was used. The reciprocal of the diagnostic real-time PCR cycle threshold value was presumed to be a good correlate of viral load. This value was multiplied by 10 to enable more convenient graphical presentation. Figure 8.5 shows the spearman correlation coefficients that describe the strength of association between viral load and cytokine/chemokine concentration. There was a moderate (r=0.5) and statistically significant positive association between the concentration of IL-6 and RSV titre. A

positive and statistically significant relationship between virus titre and cytokine/chemokine concentration was also found for IFN-γ (r=0.6, p=0.0004) and IL-10 (r=0.4, p=0.01). There was no statistically significant association between any of the remaining cytokines/chemokines and viral titre. These data are shown in figure 8.5.

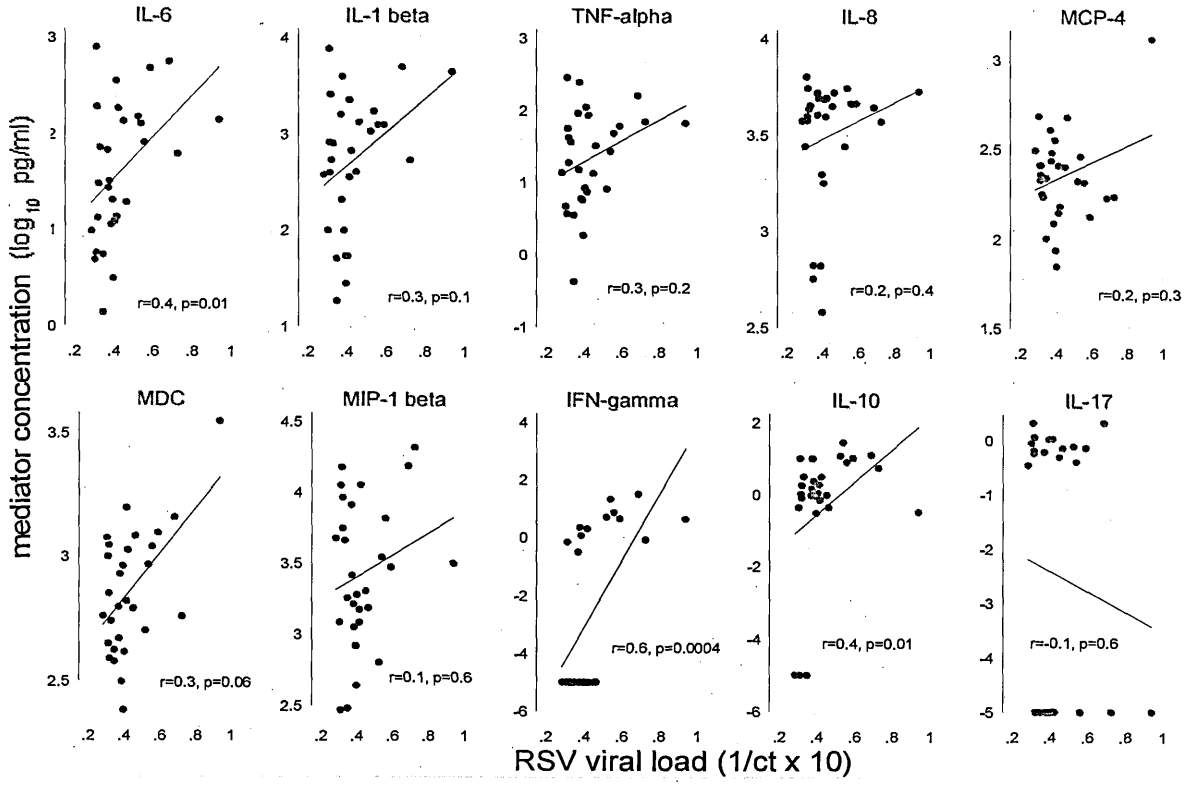


Figure 8.5 The relationship between RSV viral load and cytokine/chemokine concentration. Viral load was calculated as the inverse of cycle threshold (ct) values obtained from diagnostic real time PCR and multiplied by 10. Samples with ct values above 38 were considered to be RSV negative and were assigned a viral load value of 0 in these analyses. cytokines/chemokines that were below the limit of detection were assigned a value 0.00001pg/ml to enable log transformation for Spearman correlation analysis.

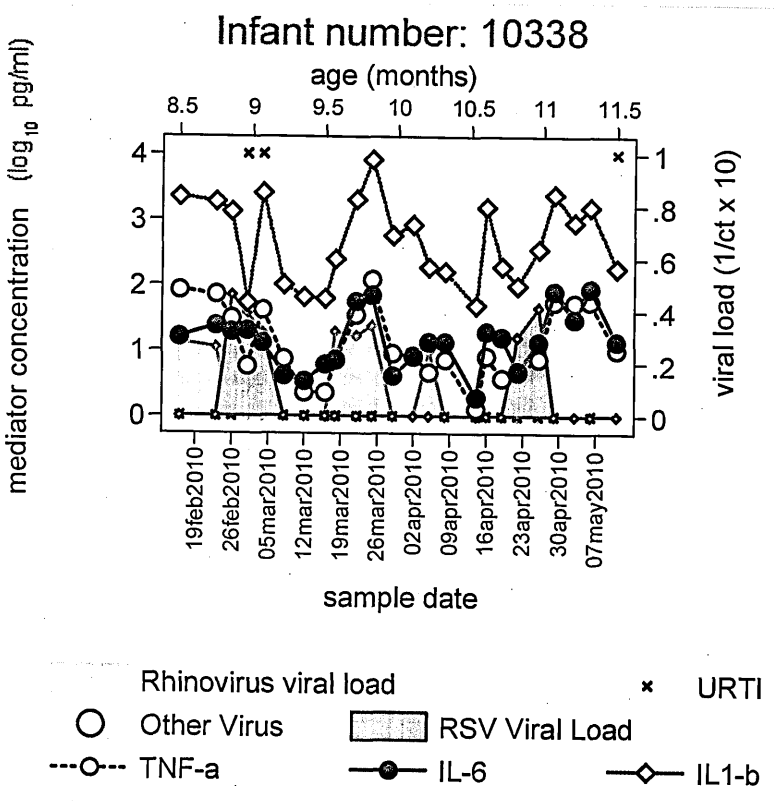
Kinetics of cytokine/chemokine responses following natural infection in infants

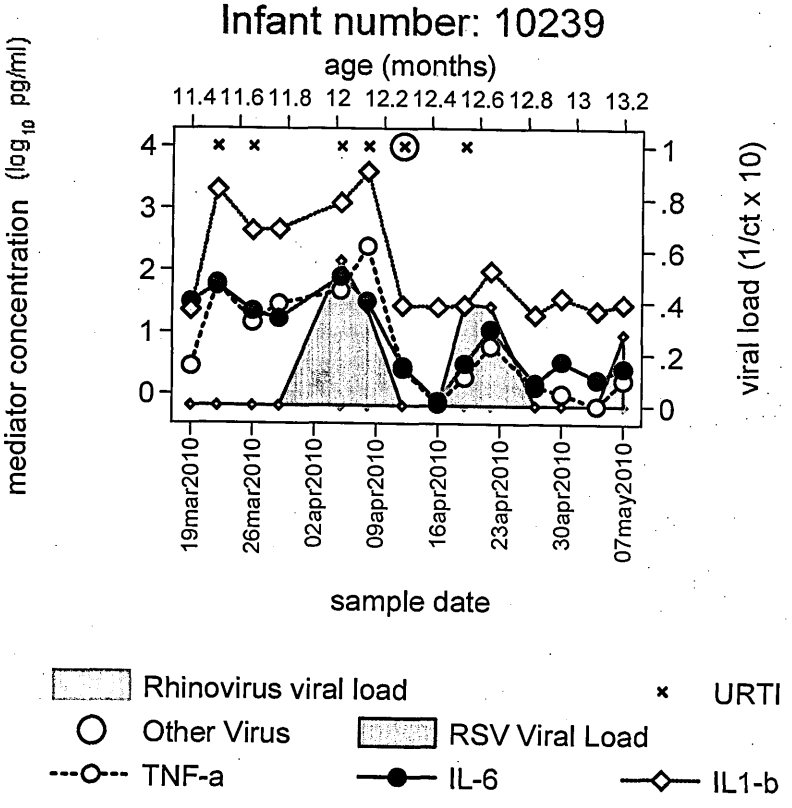
Kinetics of the pro-inflammatory cytokine/chemokine response

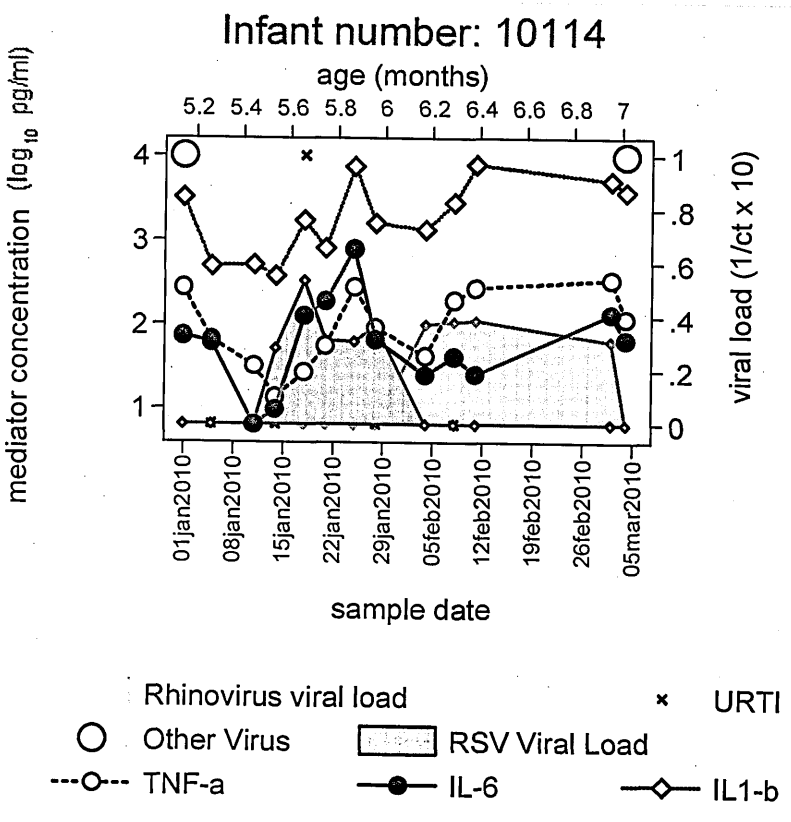
The kinetics of the pro-inflammatory cytokine/chemokine response following natural infection were evaluated by relating temporal variations in the RSV viral load to rises in the concentrations of pro-inflammatory cytokines/chemokines IL-6, TNF-α and IL1-β. The presence or absence of URTI symptoms was also related to cytokine/chemokine and viral load kinetics. For comparison the kinetics of rhinovirus viral load were included in the analysis. Figure 8.6 shows the temporal relationships between cytokine/chemokine responses, viral load and illness over the surveillance period. In general the pro-inflammatory cytokine response appeared to be closely related to the titre of both RSV and rhinovirus in infant nasal secretions. The relationship between RSV titre and the pro-inflammatory cytokine/chemokine response was most clearly evident in instances where RSV infection did not coincide with other co-infections or pre-existing URTI symptoms suggestive of an ongoing, but undetected infection. An example of this correlation can be seen in the case of infant 10182 who experienced 2 RSV infections between 25/02/2010-08/03/2010 and on 18/03/2010. In the first episode, the rise in RSV viral load coincided with an increase in the concentration of IL-6, TNF-α and IL-1β and correspondingly, the subsequent decline in viral titre was associated with a drop in concentration of these mediators. Peak RSV viral load in this initial episode was also associated with presence of URTI. In the second episode of infection, in which virus was shed for only one day there was no noticeable increase in the concentrations of these mediators. Towards the end of the surveillance period, there was a sharp rise in the

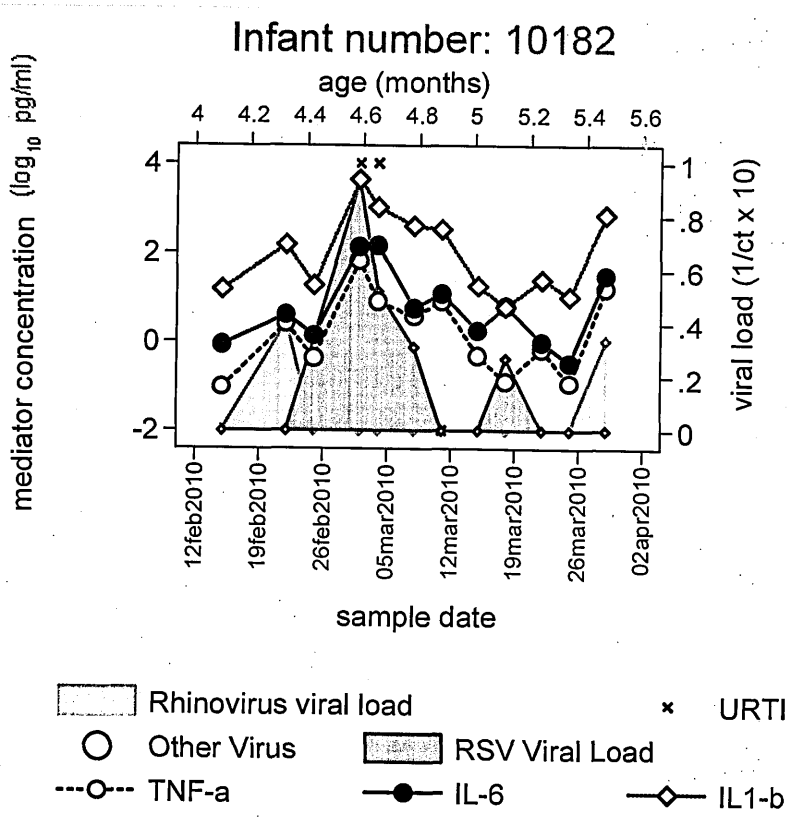
concentrations of these cytokines/chemokines that appeared to correspond with an increase in rhinovirus viral load.

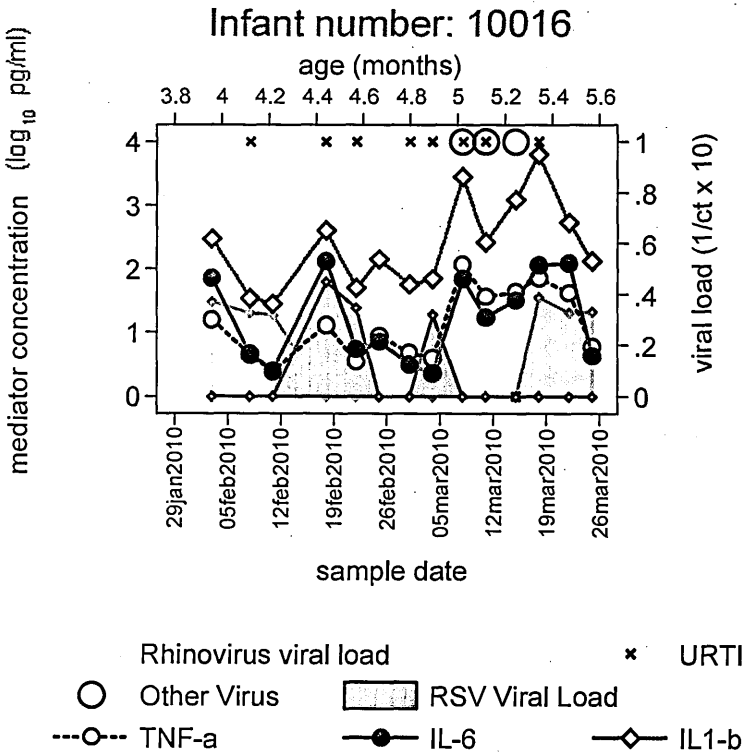
In total, a good association between RSV viral load and the pro-inflammatory response was observed in 6 of the 7 infants analysed (i.e. infants 10239, 10114, 10182, 10016 10243 and 10157). On the other hand, the relationship between these variables and disease (i.e. presence or absence of URTI) was less clearly defined. Rises in both pro-inflammatory cytokine/chemokine levels and RSV viral load clearly coincided with development of URTI in only 2 of the seven infants in this study (10114 and 10182). In the case of infants 10239, 10016, and 10243, although peak viral load and pro-inflammatory cytokine/chemokine levels were associated with presence of URTI, serial episodes of URTI were observed prior to and after RSV infection, confounding interpretation of the significance of RSV alone in development of symptoms. In the case of infant 10157, although peak cytokine/chemokine response and RSV viral load were related to the development of URTI, a simultaneous adenovirus infection was detected in the sample collected on the day in which URTI symptoms were observed. Figure 8.6 shows the relationship between cytokine/chemokine kinetics, viral load and disease.

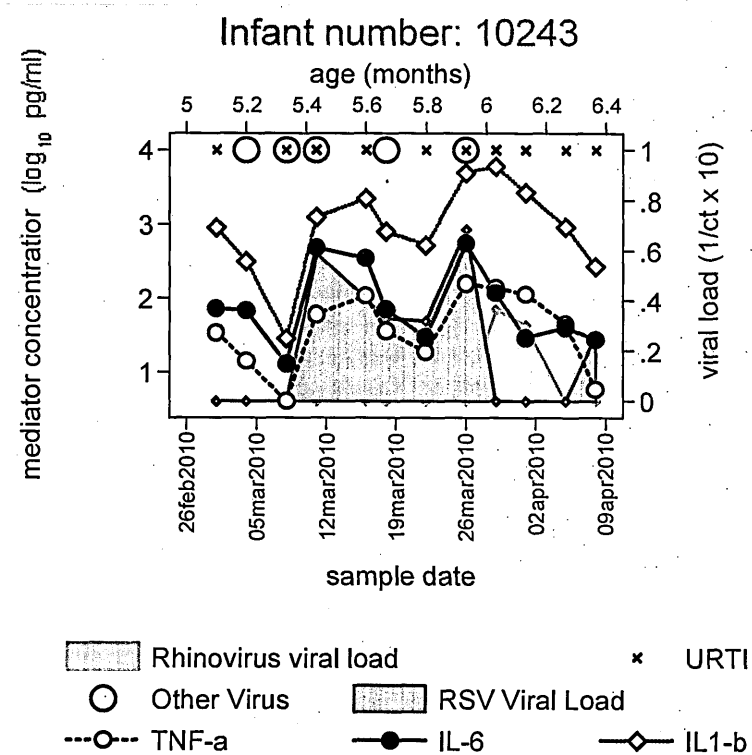

Infant number: 10338
age (months)
8.5 9 9.5 10 10.5 11 11.5
mediator concentration (log10 pg/ml)
viral load (1/ct x 10)
0 1 2 3 4
0 .2 .4 .6 .8 1
19feb2010 26feb2010 05mar2010 12mar2010 19mar2010 26mar2010 02apr2010 09apr2010 16apr2010 23apr2010 30apr2010 07may2010
sample date
Rhinovirus viral load
URTI
Other Virus
RSV Viral Load
TNF-a
IL-6
IL1-b


Infant number: 10239
age (months)
11.4 11.6 11.8 12 12.2 12.4 12.6 12.8 13 13.2
mediator concentration (log10 pg/ml)
viral load (1/ct x 10)
0 1 2 3 4
0 .2 .4 .6 .8 1
19mar2010 26mar2010 02apr2010 09apr2010 16apr2010 23apr2010 30apr2010 07may2010
sample date
Rhinovirus viral load
URTI
Other Virus
RSV Viral Load
TNF-a
IL-6
IL1-b


Infant number: 10114
age (months)
5.2 5.4 5.6 5.8 6 6.2 6.4 6.6 6.8 7
mediator concentration (log10 pg/ml)
viral load (1/ct x 10)
01jan2010
08jan2010
15jan2010
22jan2010
29jan2010
05feb2010
12feb2010
19feb2010
26feb2010
05mar2010
sample date
Rhinovirus viral load
URTI
Other Virus
RSV Viral Load
TNF-a
IL-6
IL1-b


Infant number: 10182
age (months)
4 4.2 4.4 4.6 4.8 5 5.2 5.4 5.6
mediator concentration (log10 pg/ml)
viral load (1/ct x 10)
12feb2010
19feb2010
26feb2010
05mar2010
12mar2010
19mar2010
26mar2010
02apr2010
sample date
Rhinovirus viral load
URTI
Other Virus
RSV Viral Load
TNF-a
IL-6
IL1-b


Infant number: 10016
age (months)
3.8 4 4.2 4.4 4.6 4.8 5 5.2 5.4 5.6
mediator concentration (log10 pg/ml)
viral load (1/ct x 10)
0 1 2 3 4
0 .2 .4 .6 .8 1
29jan2010 05feb2010 12feb2010 19feb2010 26feb2010 05mar2010 12mar2010 19mar2010 26mar2010
sample date
Rhinovirus viral load
URTI
Other Virus
RSV Viral Load
TNF-a
IL-6
IL1-b


Infant number: 10243
age (months)
5 5.2 5.4 5.6 5.8 6 6.2 6.4
mediator concentration (log10 pg/ml)
viral load (1/ct x 10)
1 2 3 4
0 .2 .4 .6 .8 1
26feb2010 05mar2010 12mar2010 19mar2010 26mar2010 02apr2010 09apr2010
sample date
Rhinovirus viral load
URTI
Other Virus
RSV Viral Load
TNF-a
IL-6
IL1-b

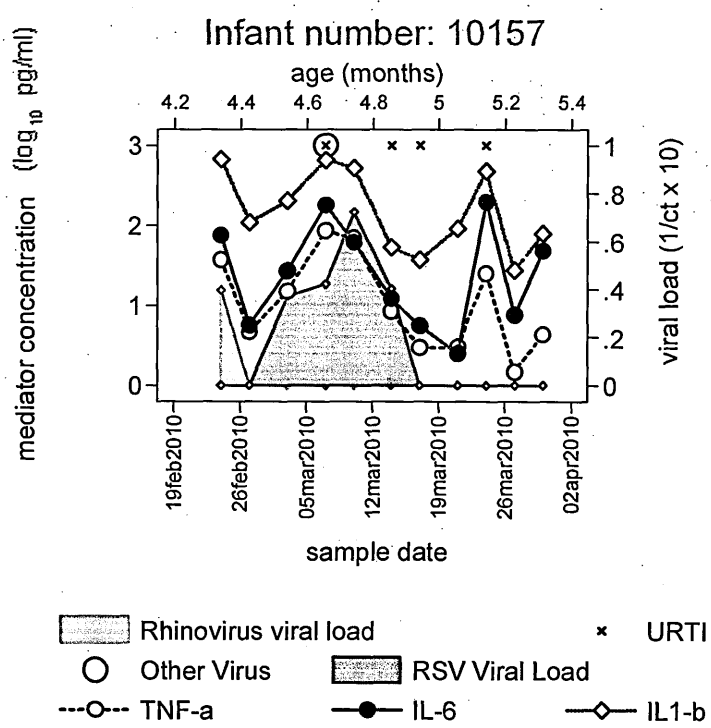
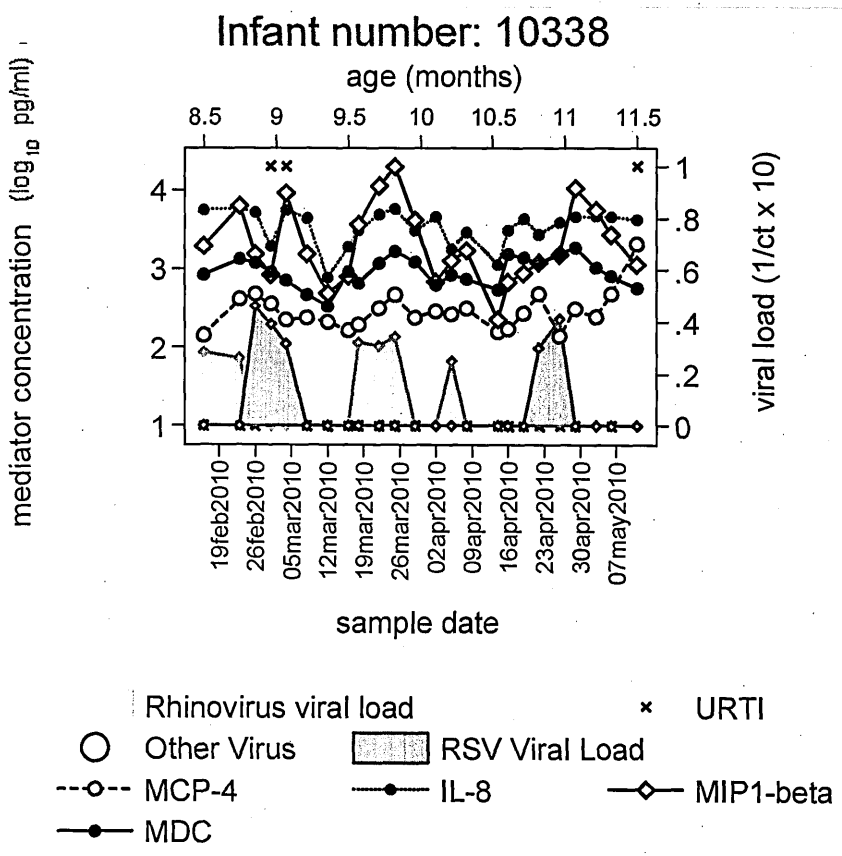


Figure 8.6 The kinetics of pro-inflammatory cytokines/chemokines in serially collected nasal secretions

Kinetics of the chemokine response

The kinetics of the chemokine response (i.e. MCP-4, IL-8, MIP-1β and MDC) appeared to be closely related with RSV/rhinovirus viral load dynamics in some infants and but not in others. For example for infant 10157, the concentrations of these chemokines appeared to initially decline with decreasing titres of rhinovirus at the beginning of the surveillance period and subsequently rose in correspondence with an increase with RSV viral load. This infant shed RSV between 03/03/2010 and 14/03/2010 and also had a concurrent adenovirus infection that was only detected in the sample collected on 07/03/2010. The dynamics of the IL-8, MDC, MCP-4 and MIP-1β responses for this infant appeared to be closely related with variations in RSV

titre since cytokine/chemokine concentrations initially rose with increasing RSV viral load and subsequently declined with decreasing virus titre. The presence of URTI was observed on 07/03/2010 while peak RSV viral load occurred on 10/03/2010. After viral clearance, a rise in the levels of IL-8, MDC and MIP-1β was detected in the sample collected on 24/03/10 despite the absence of detectable infection at this time. This rise was nonetheless associated with the presence of URTI, indicating possible presence of a viral or bacterial infection that remained undetected. In certain instances, the dynamics of the chemokine response could not be related to viral load. For instance for infant 10243, whilst the initial rise in RSV viral load was associated with a rise in the level of MIP-1β, the kinetics of the RSV viral load did not appear to correlate with variations in the concentrations of IL-8, MDC and MCP-4. Over the surveillance period highlighted in this study, this particular child had multiple Coronavirus oc43 (4/3/2010, 8/3/2010 and 11/3/2010) and nl63 (18/03/2010 and 26/03/2010) co-infections and was also found to have URTI during 10 of 12 surveillance visits, raising the possibility that an inherent susceptibility to infection might have been present in this infant. Unfortunately neither HIV nor atopic status data were available and therefore overall individual immune status could not be assessed in this study. The dynamics of the chemokine response for the remaining infants fell broadly within these two categories and are shown in figure 8.7.


Infant number: 10338
age (months)
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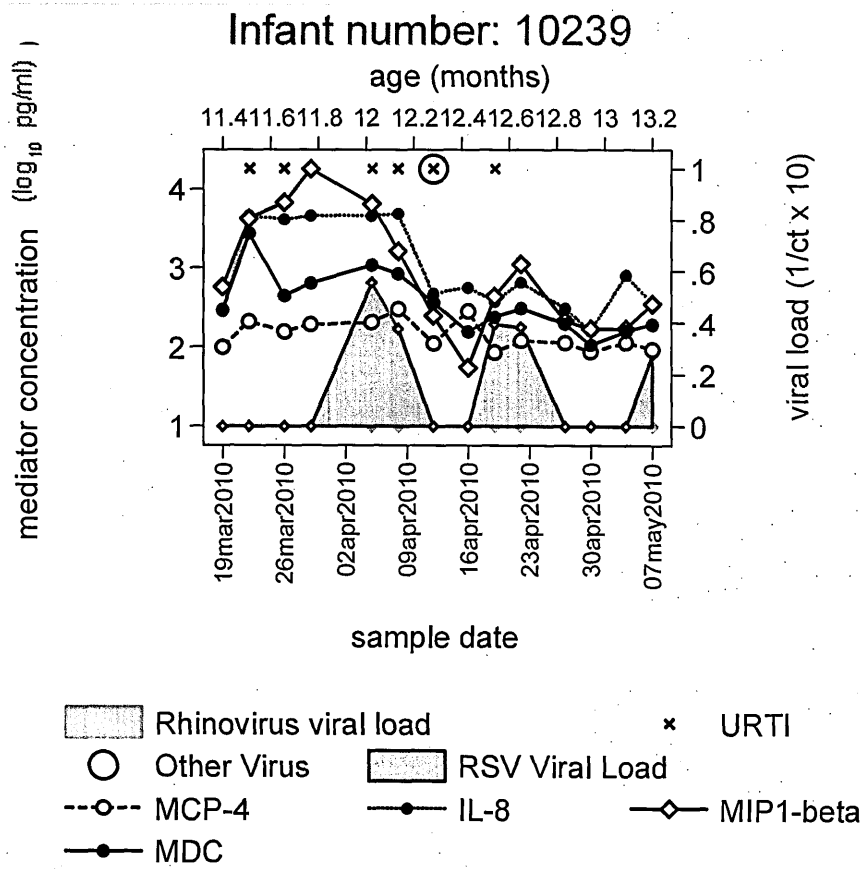
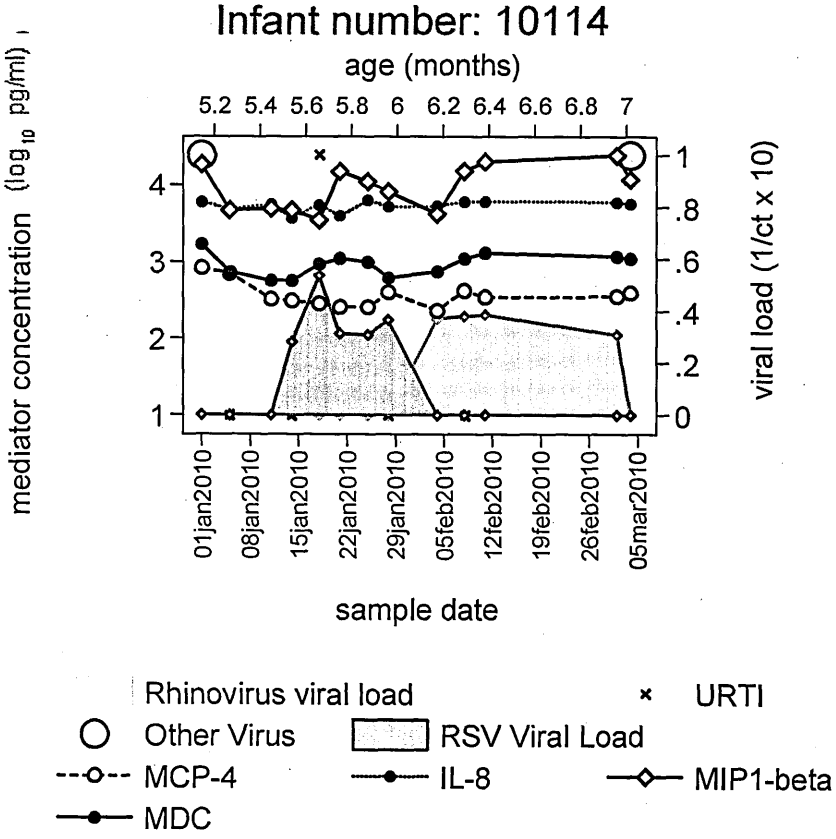
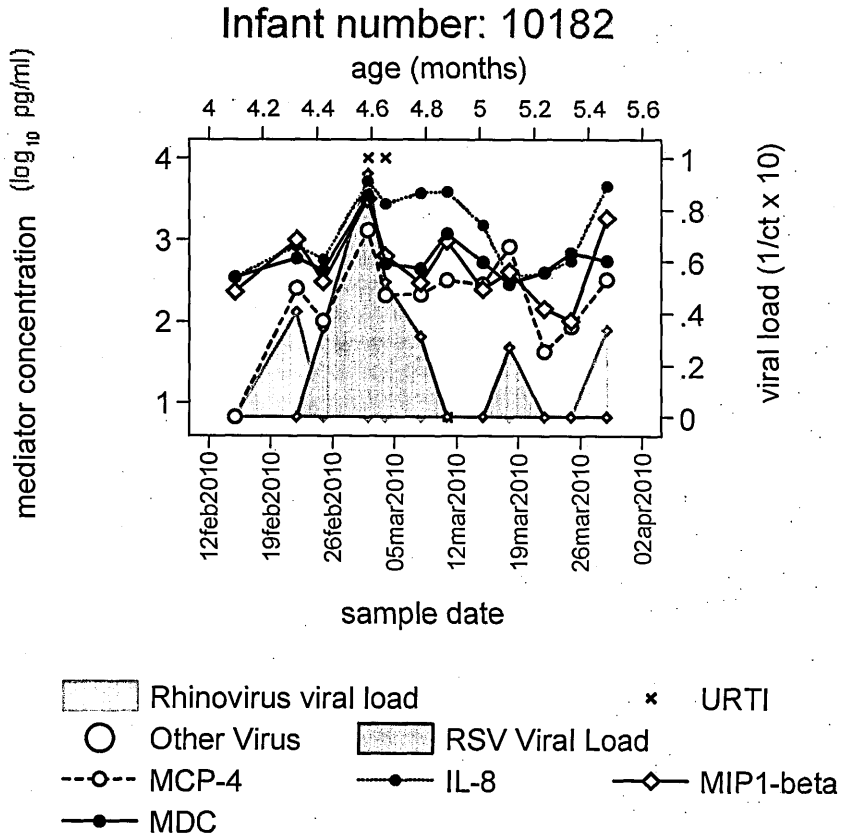
mediator concentration (log10 pg/ml)
viral load (1/ct x 10)
19feb2010
26feb2010
05mar2010
12mar2010
19mar2010
26mar2010
02apr2010
09apr2010
16apr2010
23apr2010
30apr2010
07may2010
sample date
Rhinovirus viral load
URTI
Other Virus
RSV Viral Load
MCP-4
IL-8
MIP1-beta
MDC


Infant number: 10239
age (months)
11.4 11.6 11.8 12 12.2 12.4 12.6 12.8 13 13.2
mediator concentration (log10 pg/ml)
viral load (1/ct x 10)
19mar2010
26mar2010
02apr2010
09apr2010
16apr2010
23apr2010
30apr2010
07may2010
sample date
Rhinovirus viral load
URTI
Other Virus
RSV Viral Load
MCP-4
IL-8
MIP1-beta
MDC


Infant number: 10114
age (months)
5.2 5.4 5.6 5.8 6 6.2 6.4 6.6 6.8 7
mediator concentration (log_{10} pg/ml)
viral load (1/ct x 10)
01jan2010
08jan2010
15jan2010
22jan2010
29jan2010
05feb2010
12feb2010
19feb2010
26feb2010
05mar2010
sample date
Rhinovirus viral load
URTI
Other Virus
RSV Viral Load
MCP-4
IL-8
MIP1-beta
MDC


Infant number: 10182
age (months)
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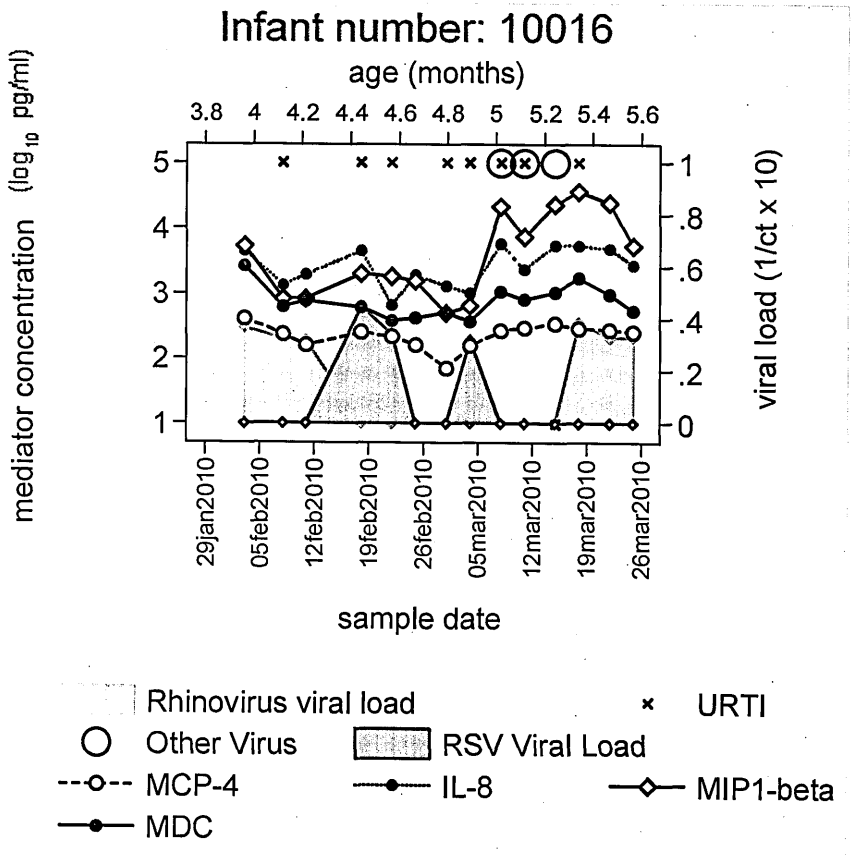
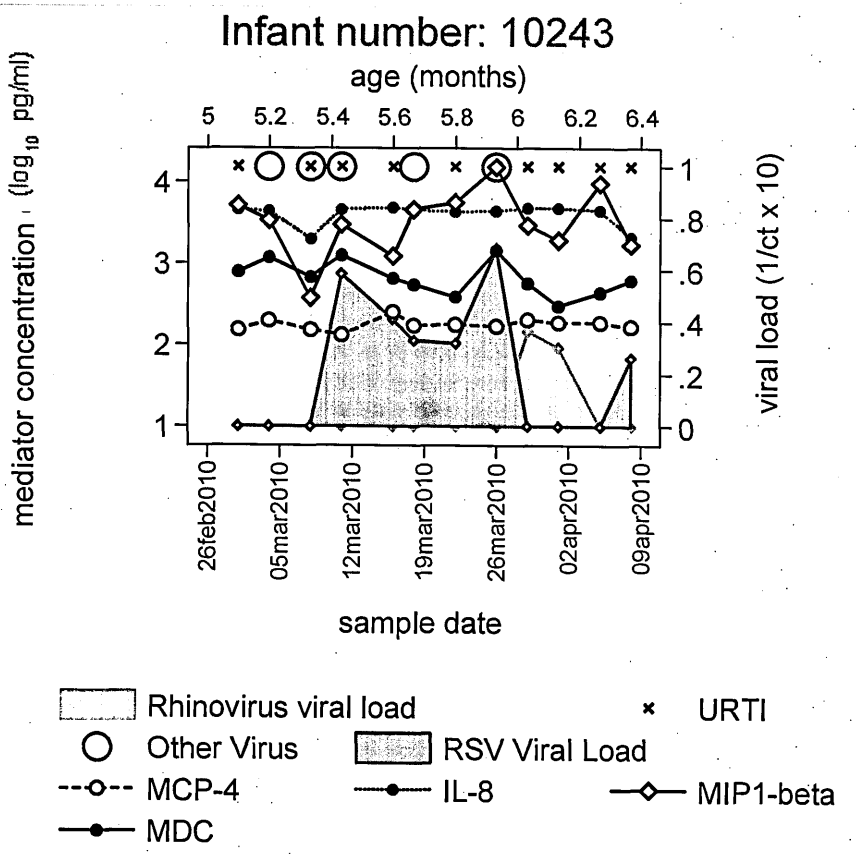
mediator concentration (log_{10} pg/ml)
viral load (1/ct x 10)
12feb2010
19feb2010
26feb2010
05mar2010
12mar2010
19mar2010
26mar2010
02apr2010
sample date
Rhinovirus viral load
URTI
Other Virus
RSV Viral Load
MCP-4
IL-8
MIP1-beta
MDC


Infant number: 10016
age (months)
3.8 4 4.2 4.4 4.6 4.8 5 5.2 5.4 5.6
mediator concentration (log10 pg/ml)
viral load (1/ct x 10)
29jan2010
05feb2010
12feb2010
19feb2010
26feb2010
05mar2010
12mar2010
19mar2010
26mar2010
sample date
Rhinovirus viral load
URTI
Other Virus
RSV Viral Load
MCP-4
IL-8
MIP1-beta
MDC


Infant number: 10243
age (months)
5 5.2 5.4 5.6 5.8 6 6.2 6.4
mediator concentration (log10 pg/ml)
viral load (1/ct x 10)
26feb2010
05mar2010
12mar2010
19mar2010
26mar2010
02apr2010
09apr2010
sample date
Rhinovirus viral load
URTI
Other Virus
RSV Viral Load
MCP-4
IL-8
MIP1-beta
MDC

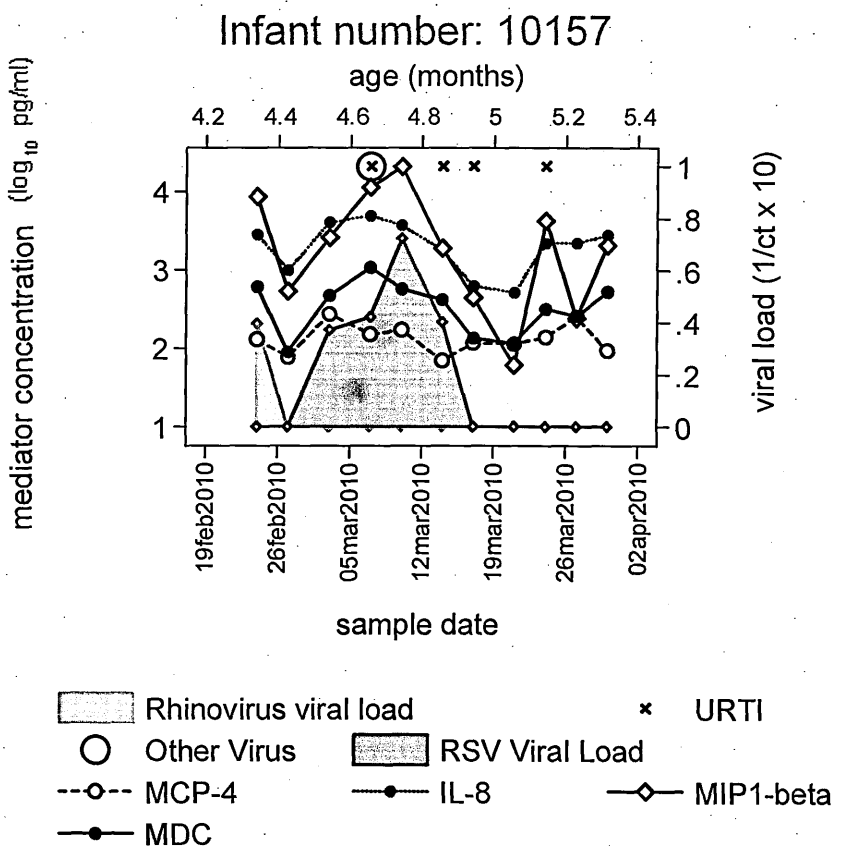
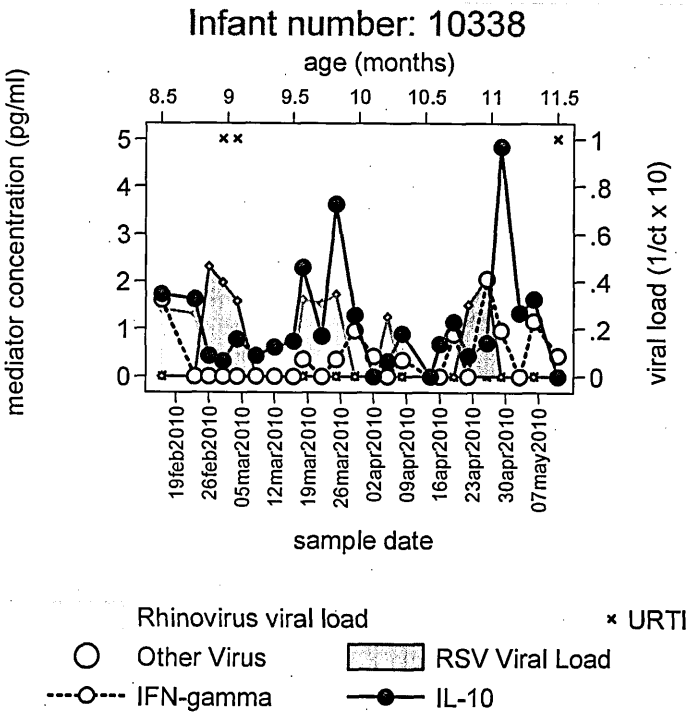


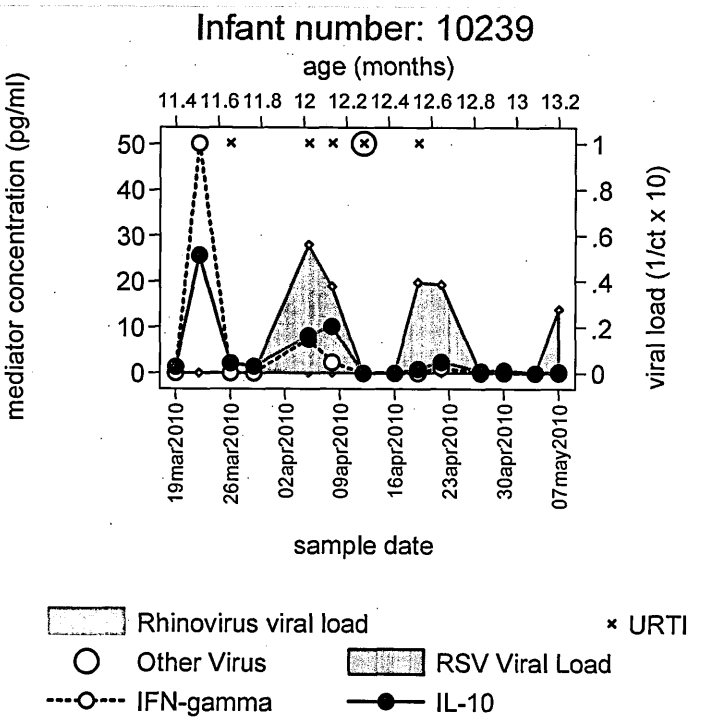
Figure 8.7 The kinetics of different chemokines in serially collected nasal secretions

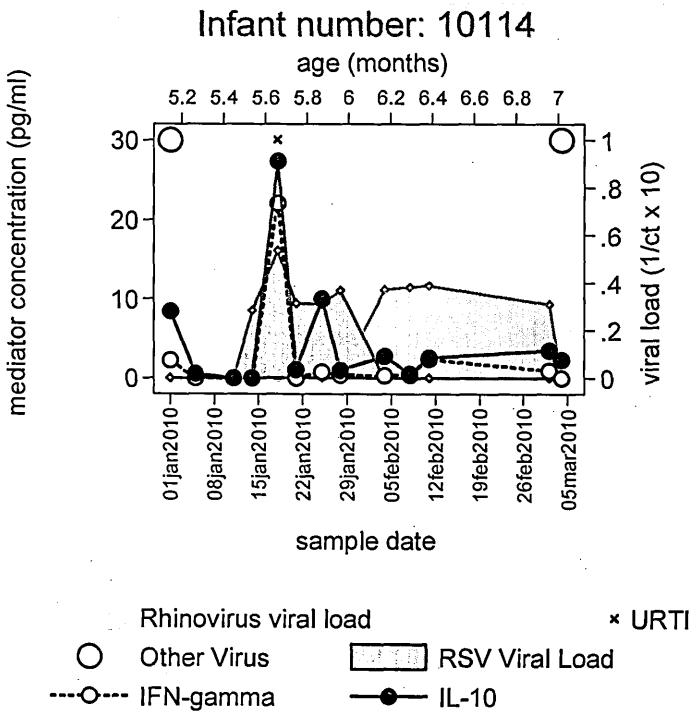
Kinetics of the IFN-γ and IL-10 response

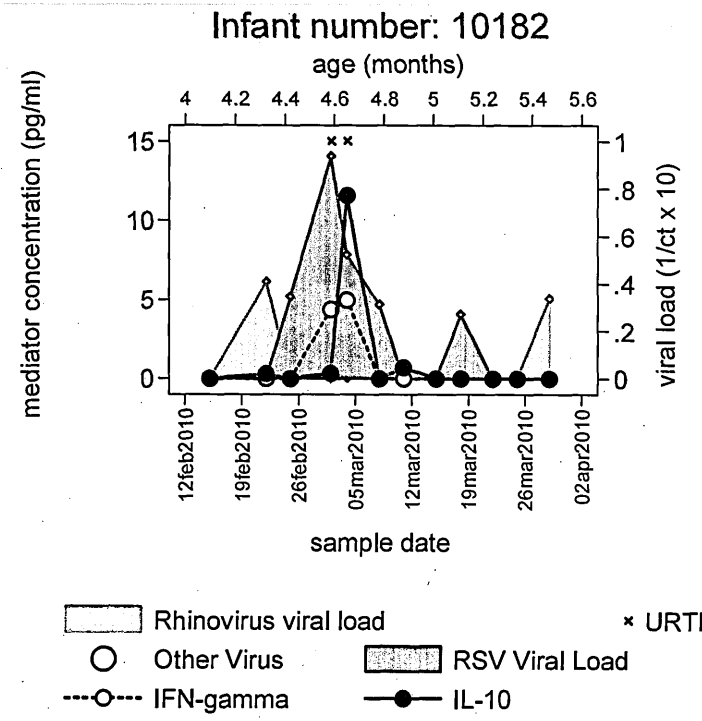
In order to assess the balance between pro-inflammatory and regulatory responses at the initial stages of the host antiviral response, the kinetics of the IFN-γ response to infection were compared to those of the IL-10 response. For most infants in this study, there was strong evidence of co-ordinated production of IFN-γ and IL-10 following natural infection. The data presented in figure 8.8 highlight the kinetics of these two cytokines in the nasal secretions of infants in this study. For most infants peak levels of these cytokines coincided with peak viral load or occurred shortly afterwards. This strong relationship between peak viral titre and peak IFN-γ/IL-10 response was seen

in the initial RSV infections of 5 of the 7 infants in this study: 10239, 10114, 10182, 10243 and 10157. In the case of infant 10338 who had two distinct RSV infections, this relationship was seen during the second infection. Two of the 7 infants in this study (10239 and 10157) had increases in IFN-γ/IL-10 levels in the absence of detectable infection, raising the possibility that they had been infected with respiratory pathogens that were not part of the panel that was detected. Of the 5 infants for whom peak RSV viral load correlated with peak IFN-γ and IL-10 concentrations, the concentrations of IL-10 were greater than those of IFN-γ for 4 of these infants (10114, 10182, 10243 and 10157). However, for infant 10243 who appeared to have acquired a re-infection shortly after the initial infection the pattern of the IL-10/IFN-γ response was reversed in the second infection. For two of the seven infants, some RSV infection events could not be clearly related to strong IFN-γ/IL-10 responses. The first infection experienced by infant 10338 (between 26/02/2010 and 04/03/2010) was not associated with clear increases in the levels of either IL-10 or IFN-γ. However, the second infection (between 22/04/2010 and 26/04/2010) appeared to induce increases in the levels of both cytokines. In the case of infant 10016, the initial rise in RSV titre (between 18/02/2010 and 22/02/2010) was associated with a modest increase in the level of IL-10, but no increase in the level of IFN-γ. The second peak, which was only detected on 04/03/2010 was not associated with a clear rise in the concentration of either cytokine. Figure 8.8 shows the dynamics of IFN-γ and IL-10 responses in the samples of the seven infants in this study.


Infant number: 10338
age (months)
8.5 9 9.5 10 10.5 11 11.5
mediator concentration (pg/ml)
viral load (1/ct x 10)
0 1 2 3 4 5
0 .2 .4 .6 .8 1
19feb2010 26feb2010 05mar2010 12mar2010 19mar2010 26mar2010 02apr2010 09apr2010 16apr2010 23apr2010 30apr2010 07may2010
sample date
Rhinovirus viral load
× URTI
Other Virus
RSV Viral Load
IFN-gamma
IL-10


Infant number: 10239
age (months)
11.4 11.6 11.8 12 12.2 12.4 12.6 12.8 13 13.2
mediator concentration (pg/ml)
viral load (1/ct x 10)
0 10 20 30 40 50
0 .2 .4 .6 .8 1
19mar2010 26mar2010 02apr2010 09apr2010 16apr2010 23apr2010 30apr2010 07may2010
sample date
Rhinovirus viral load
× URTI
Other Virus
RSV Viral Load
IFN-gamma
IL-10


Infant number: 10114
age (months)
5.2 5.4 5.6 5.8 6 6.2 6.4 6.6 6.8 7
mediator concentration (pg/ml)
30
20
10
0
viral load (1/ct x 10)
1
.8
.6
.4
.2
0
01jan2010
08jan2010
15jan2010
22jan2010
29jan2010
05feb2010
12feb2010
19feb2010
26feb2010
05mar2010
sample date
Rhinovirus viral load
× URTI
Other Virus
RSV Viral Load
IFN-gamma
IL-10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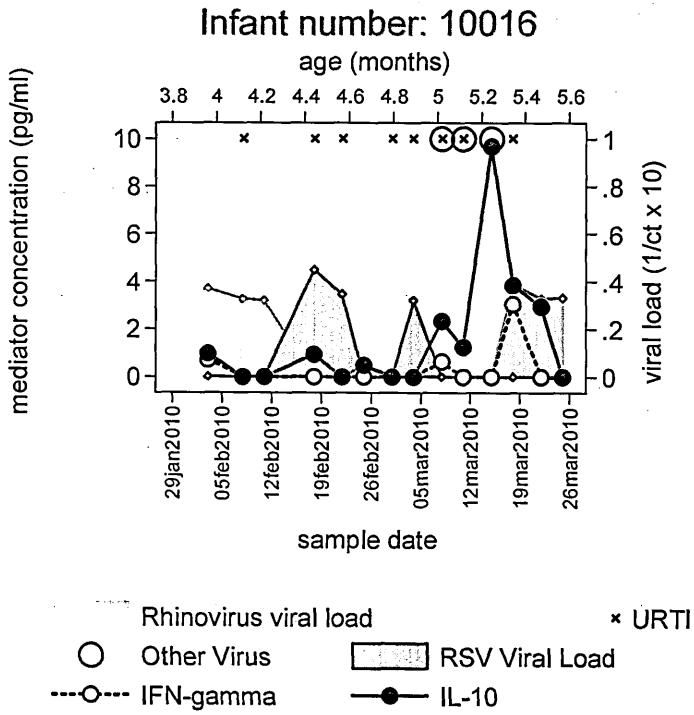
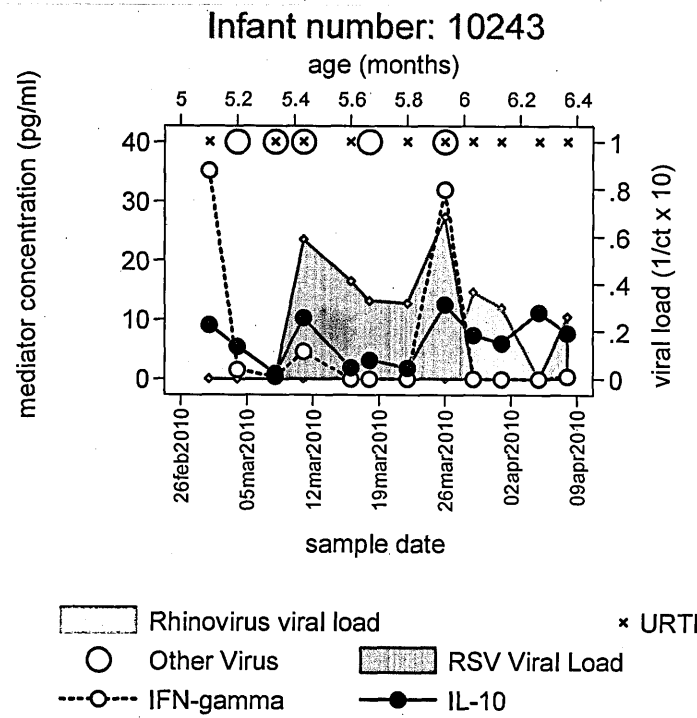
age (months)
4 4.2 4.4 4.6 4.8 5 5.2 5.4 5.6
mediator concentration (pg/ml)
15
10
5
0
viral load (1/ct x 10)
1
.8
.6
.4
.2
0
12feb2010
19feb2010
26feb2010
05mar2010
12mar2010
19mar2010
26mar2010
02apr2010
sample date
Rhinovirus viral load
× URTI
Other Virus
RSV Viral Load
IFN-gamma
IL-10


Infant number: 10016
age (months)
3.8 4 4.2 4.4 4.6 4.8 5 5.2 5.4 5.6
mediator concentration (pg/ml)
viral load (1/ct x 10)
0 2 4 6 8 10
0 .2 .4 .6 .8 1
29jan2010 05feb2010 12feb2010 19feb2010 26feb2010 05mar2010 12mar2010 19mar2010 26mar2010
sample date
Rhinovirus viral load
× URTI
Other Virus
RSV Viral Load
IFN-gamma
IL-10


Infant number: 10243
age (months)
5 5.2 5.4 5.6 5.8 6 6.2 6.4
mediator concentration (pg/ml)
viral load (1/ct x 10)
0 10 20 30 40
0 .2 .4 .6 .8 1
26feb2010 05mar2010 12mar2010 19mar2010 26mar2010 02apr2010 09apr2010
sample date
Rhinovirus viral load
× URTI
Other Virus
RSV Viral Load
IFN-gamma
IL-10

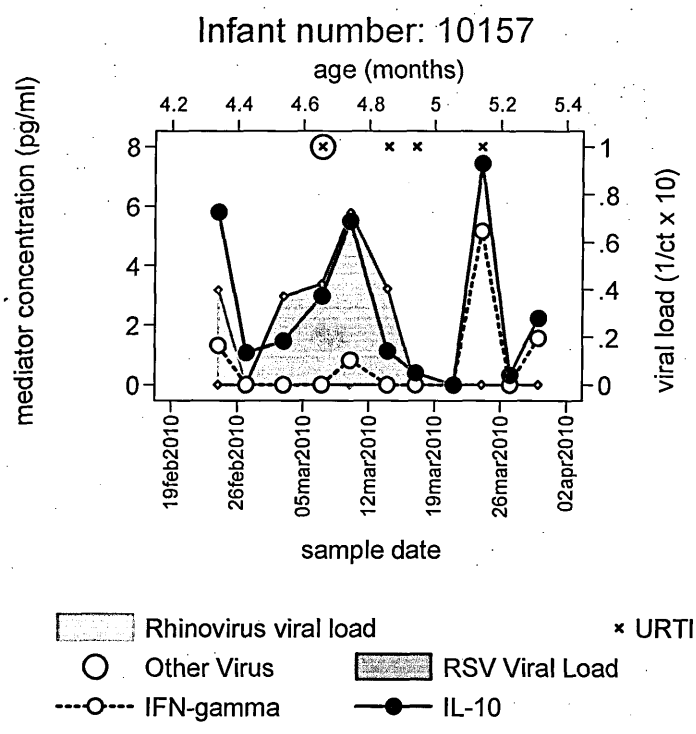
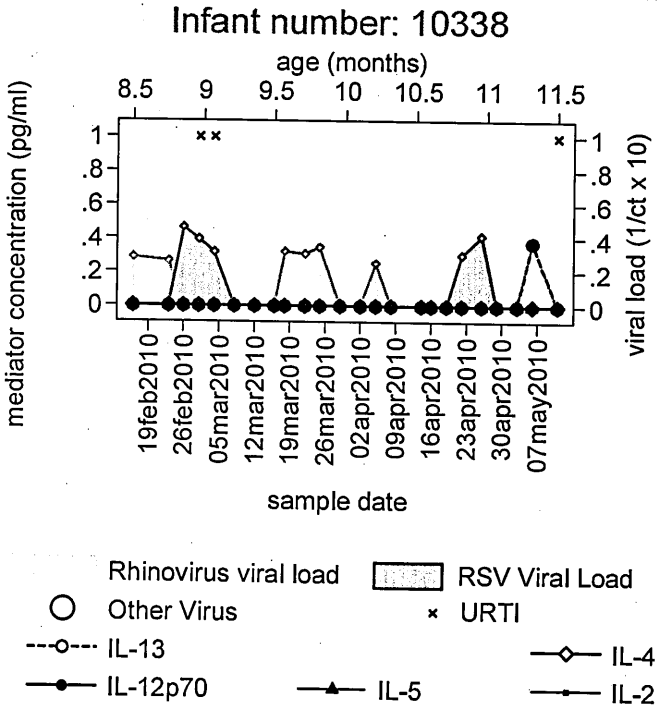


Figure 8.8 The kinetics of IFN-γ and IL-10 in serially collected nasal secretions

Kinetics of T cell associated mediators

Cytokines associated with T lymphocytes (IL-2, IL-4, IL-5, IL-12p70 and IL-13) were generally not detectable throughout natural RSV infection. As is evident from figure 8.9, except for the second RSV peak in infant 10243, no other infant produced high levels of these cytokines. For infant 10243, the rise in T cell associated cytokines in the sample collected on 26/03/2010, coincided with both an RSV and coronavirus nl63 infection.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age (months)
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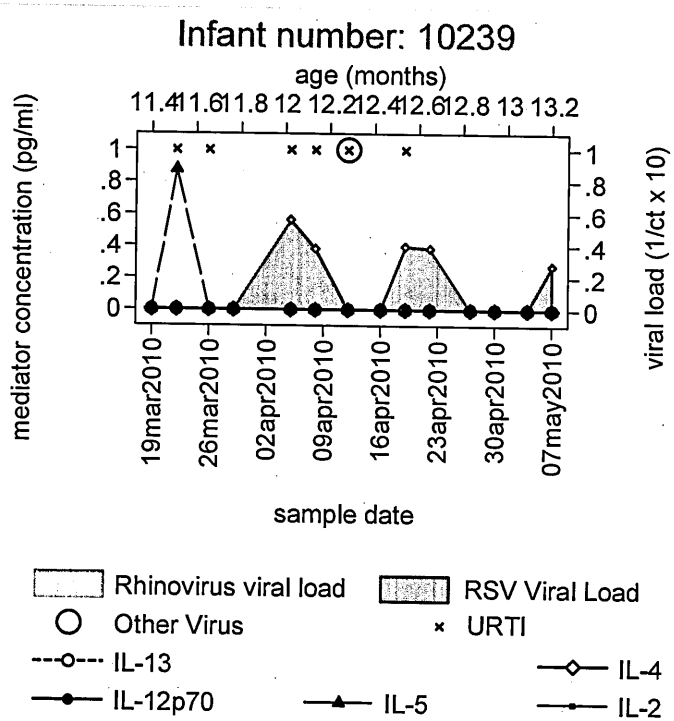
mediator concentration (pg/ml)
viral load (1/ct x 10)
0 .2 .4 .6 .8 1
19feb2010 26feb2010 05mar2010 12mar2010 19mar2010 26mar2010 02apr2010 09apr2010 16apr2010 23apr2010 30apr2010 07may2010
sample date
Rhinovirus viral load
RSV Viral Load
Other Virus
URTI
IL-13
IL-4
IL-12p70
IL-5
IL-2


Infant number: 10239
age (months)
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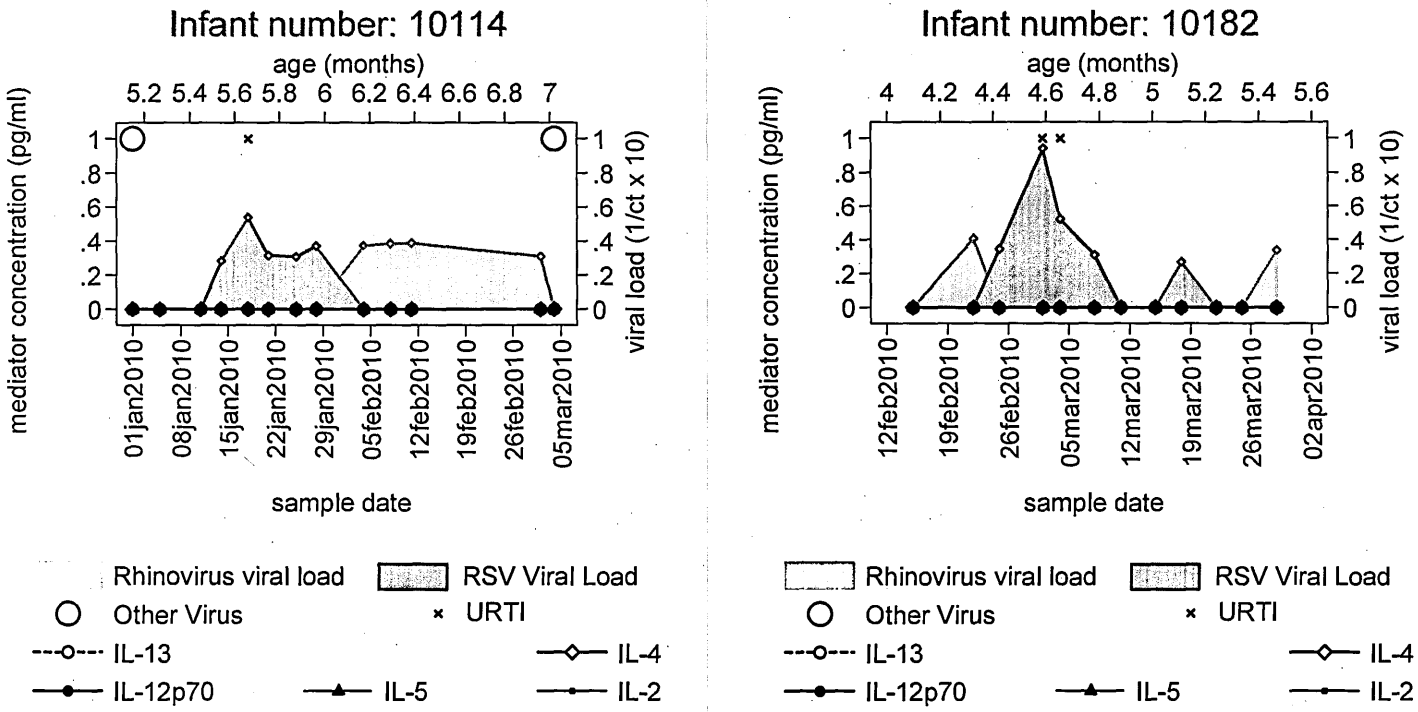
mediator concentration (pg/ml)
viral load (1/ct x 10)
0 .2 .4 .6 .8 1
19mar2010 26mar2010 02apr2010 09apr2010 16apr2010 23apr2010 30apr2010 07may2010
sample date
Rhinovirus viral load
RSV Viral Load
Other Virus
URTI
IL-13
IL-4
IL-12p70
IL-5
IL-2


Infant number: 10114
age (months)
5.2 5.4 5.6 5.8 6 6.2 6.4 6.6 6.8 7
mediator concentration (pg/ml)
viral load (1/ct x 10)
01jan2010
08jan2010
15jan2010
22jan2010
29jan2010
05feb2010
12feb2010
19feb2010
26feb2010
05mar2010
sample date
Rhinovirus viral load
RSV Viral Load
Other Virus
URTI
IL-13
IL-4
IL-12p70
IL-5
IL-2

Infant number: 10182
age (months)
4 4.2 4.4 4.6 4.8 5 5.2 5.4 5.6
mediator concentration (pg/ml)
viral load (1/ct x 10)
12feb2010
19feb2010
26feb2010
05mar2010
12mar2010
19mar2010
26mar2010
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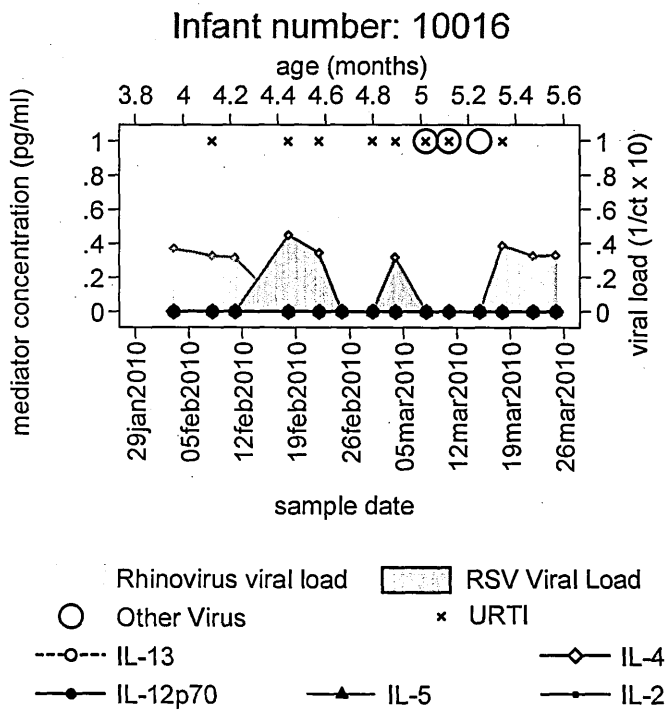
RSV Viral Load
Other Virus
URTI
IL-13
IL-4
IL-12p70
IL-5
IL-2


Infant number: 10016
age (months)
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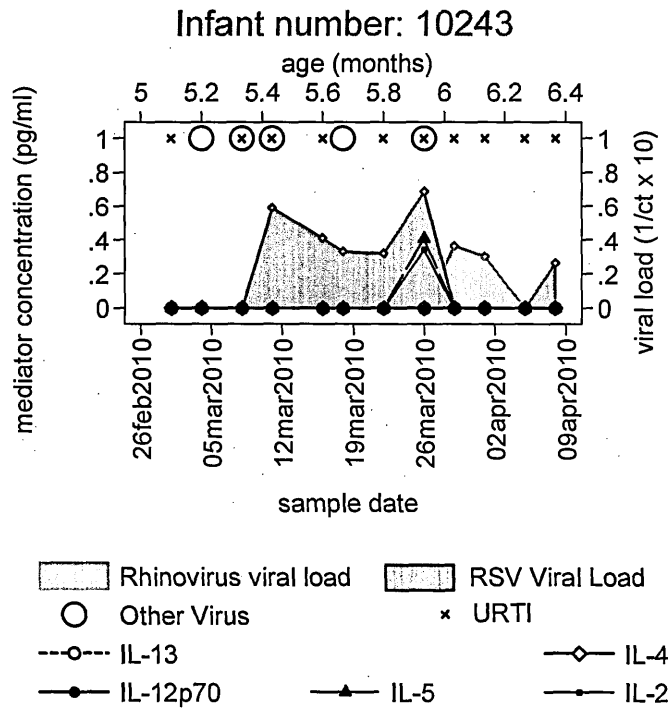
mediator concentration (pg/ml)
viral load (1/ct x 10)
0 .2 .4 .6 .8 1
29jan2010 05feb2010 12feb2010 19feb2010 26feb2010 05mar2010 12mar2010 19mar2010 26mar2010
sample date
Rhinovirus viral load
RSV Viral Load
Other Virus
URTI
IL-13
IL-4
IL-12p70
IL-5
IL-2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age (months)
5 5.2 5.4 5.6 5.8 6 6.2 6.4
mediator concentration (pg/ml)
viral load (1/ct x 10)
0 .2 .4 .6 .8 1
26feb2010 05mar2010 12mar2010 19mar2010 26mar2010 02apr2010 09apr2010
sample date
Rhinovirus viral load
RSV Viral Load
Other Virus
URTI
IL-13
IL-4
IL-12p70
IL-5
IL-2

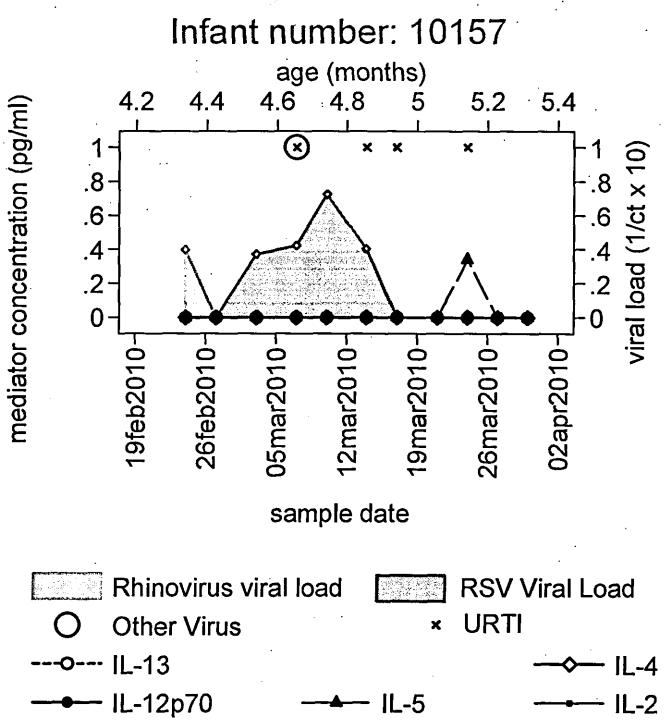
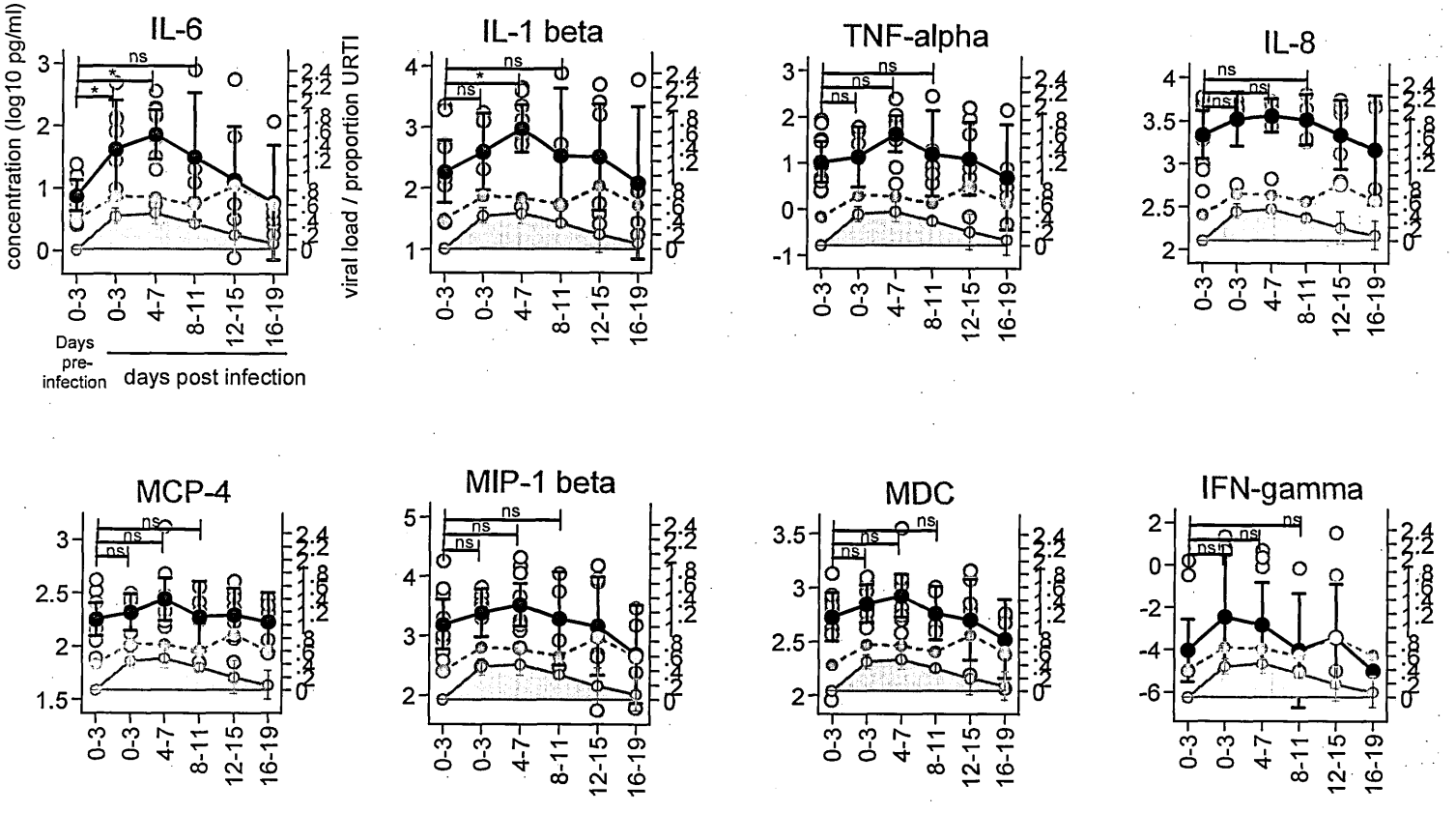


Figure 8.9 The kinetics of T cell cytokines/chemokines in serially collected nasal secretions

Summary of cytokine/chemokine responses in the household study

Summary cytokine/chemokine profiles were generated using mean cytokine/chemokine concentrations at different time points pre- and post-infection. Infants from whom RSV negative samples were collected approximately 3 days prior to the collection date of an RSV positive sample were considered to have acquired infection between 0-3 days after collection of the RSV negative samples. Similarly, infants from whom RSV positive samples were collected approximately 3 days after collection of RSV negative samples, were considered to have acquired infection between 0-3 days prior to the collection of the RSV positive samples. The mean cytokine/chemokine concentrations were measured at 6 time points; 0-3 days pre-

infection, 0-3, 4-7, 8-11, 12-15 and 16-19 days post infection. Mean viral load as well as the proportion of infants who had URTI at these time points were calculated and plotted relative to cytokine/chemokine concentrations as shown in figure 8.10. Induction and maintenance of cytokine/chemokine responses following natural infection was assessed by comparing the mean cytokine/chemokine concentrations at different time points post infection, to the mean cytokine/chemokine concentrations at between 0-3 days pre-infection. There was a statistically significant increase in the concentration of IL-6 at between 0-3 days post infection ($p=0.04$) as well as between 4-7 days post infection ($p=0.003$) relative to the pre-infection control. The mean concentration of IL-6 at between 8-11 days post infection, was no different from the mean pre-infection control concentration ($p=0.1$). The proportion of infants who had URTI at between 0-3 days pre-infection was 40%, while the proportion of URTI at 0-3, 4-7, 8-11, 12-15 and 16-19 days post infection was 60%, 70%, 71% and 86%, respectively. The mean concentration of IL-1β at between 0-3 days post infection, was similar to the mean pre-infection control concentration ($p=0.4$), but was significantly raised at between 4-7 days post infection ($p=0.047$). There was no significant difference between the mean concentration of IL-1β at between 8-11 days post infection and the mean pre-infection control concentration ($p=0.5$). In the case of all other mediators, there was no statistically significant difference between mean cytokine/chemokine concentration in the pre-infection control and mean cytokine/chemokine concentrations at between 0-3, 4-7 and 8-11 days post infection. Figure 8.10 is a summary of these data.


IL-6
IL-1 beta
TNF-alpha
IL-8
MCP-4
MIP-1 beta
MDC
IFN-gamma
concentration (log10 pg/ml)
viral load / proportion URTI
Days pre-infection
days post infection
0-3
4-7
8-11
12-15
16-19
ns
*

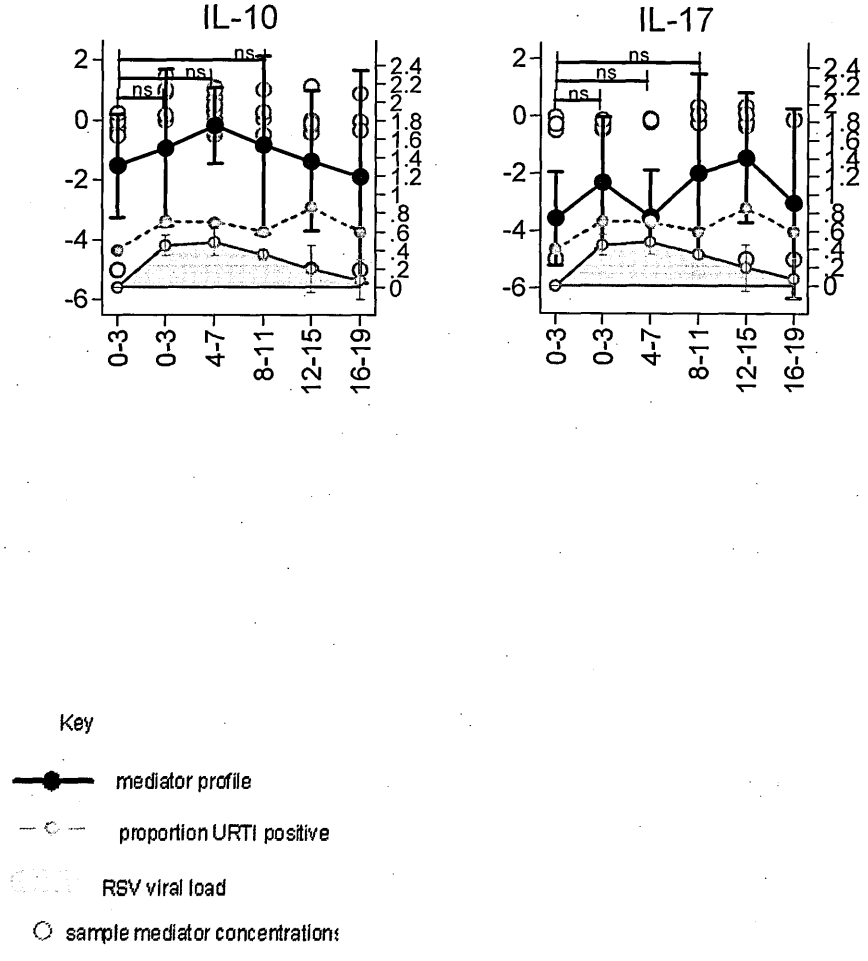


Figure 8.10 Comparison of mean cytokine/chemokine concentrations at different time points pre- and post-infection

Mediator responses in nasal secretions of infants admitted with RSV associated severe/ very severe pneumonia.

RSV group-specific cytokine/chemokine responses

In order to determine whether virus-specific factors contribute to variations in the cytokine/chemokine response, the levels of different cytokines/chemokines in infants infected with RSV A or B and who had been admitted with severe RSV-associated pneumonia were compared. There were no statistically significant differences in the levels of IL-1β, IL-6, IL-8, TNF-α, MDC, MIP-1β, IFN-γ, IL-10 and IL-17 in nasal samples obtained from RSV A or RSV B infected infants. However, there was a statistically significant difference between the levels of MCP-4 in the nasal secretions of RSV A and RSV B infected infants ($p=0.005$) with RSV A being associated with higher concentrations of this cytokine/chemokine relative to RSV B. These data are graphically shown in figure 8.11.

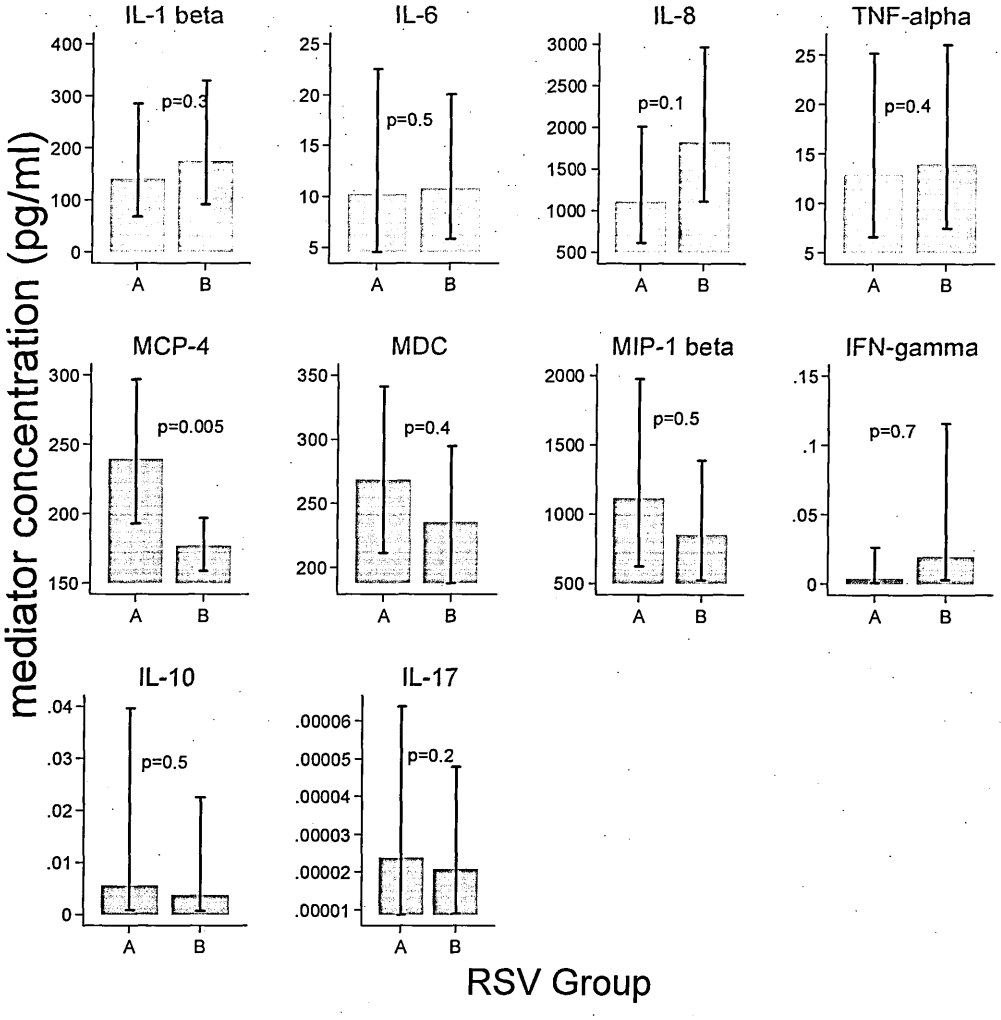


Figure 8.11 Comparison of mean cytokine/chemokine concentrations in nasal secretions from which RSV A (n=42) or RSV B (n=44) was isolated

Association between cytokine/chemokine responses and severe or very severe pneumonia

The role of different cytokines/chemokines in modifying the clinical phenotype of RSV associated severe pneumonia was explored by comparing cytokine/chemokine concentrations between infants admitted with WHO defined severe (n=49) or very severe (n=29) pneumonia. Only 8 infants had mild pneumonia and were not included in the analysis. The levels of IL-1β, IL-6, IL-8, IL-8, TNF-α, MCP-4, MDC, MIP-1β,

IFN-γ, IL-10, and IL-17 did not vary irrespective of the clinical phenotype of disease. These data are graphically presented in figure 8.12.

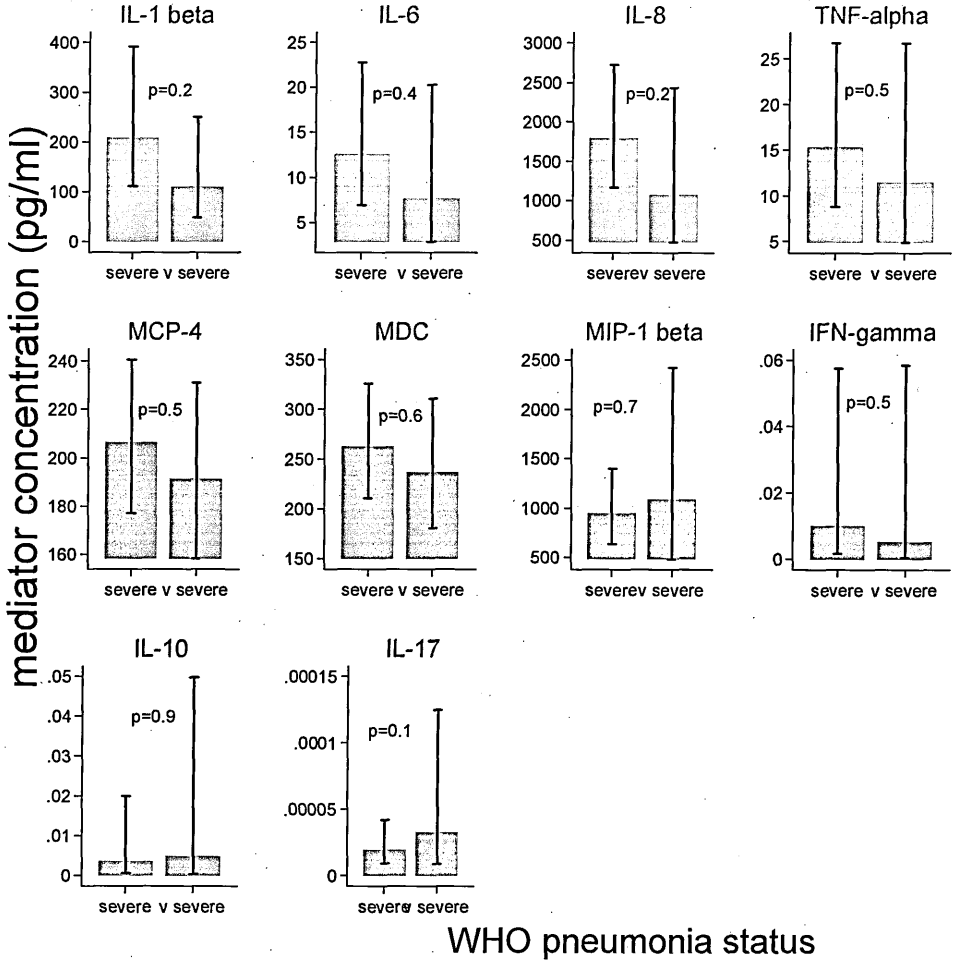


Figure 8.12 Comparison of mean cytokine/chemokine concentrations in nasal secretions obtained from infants admitted with severe (n=49) or very severe (n=29) pneumonia

Discussion

This study investigated the natural history of the cytokine/chemokine response over the course of natural RSV infection in infants. Analysis of cytokine/chemokine responses in the presence of RSV or rhinovirus infection showed that in general,

rhinovirus infection was associated with higher concentrations of a number of cytokines/chemokines relative to RSV. The levels of IL-1β, TNF-α, IL-8, MIP-1β and MDC were all significantly higher in the presence of rhinovirus infection. One possible reason for the discrepancy between RSV and rhinovirus specific cytokine/chemokine responses may be differential abilities to antagonise the host antiviral response. RSV has evolved mechanisms such as interferon antagonism by non-structural proteins NS1 and NS2 (Schlender *et al.*, 2000, Lo *et al.*, 2005), that are thought to subvert the host antiviral response and consequently delay viral clearance. Comparison of cytokine/chemokine concentrations produced following natural influenza and RSV infection shows that RSV infection generally induces significantly lower cytokine/chemokine concentrations relative to influenza (Welliver *et al.*, 2007). The fact that both rhinovirus and influenza infection (both associated with reduced clinical pathology in infants relative to RSV) generally induce cytokine/chemokine responses at higher levels relative to RSV, suggests that antagonism of cytokine/chemokine responses may be a mechanism that is related to development of severe RSV disease in infants. Recent work has shown that this interferon antagonism has profound downstream effects on multiple host immune functions such as the suppression of dendritic cell maturation (Munir *et al.*, 2008) and decreased activation of T cells (Munir *et al.*, 2011). It is possible that the observed results could be explained by the ability of RSV to antagonise host cytokine/chemokine responses, with the ultimate goal of prolonging viral replication.

The role of viral load in driving RSV pathogenesis has been the subject of many controversial studies. While some investigators have found that viral titres at the acute stage of illness are significantly associated with disease severity (DeVincenzo *et al.*,

2005), others have failed to find such an association (Wright *et al.*, 2002). In many studies, this association is often evaluated at the time of clinical presentation, which often coincides with the peak of illness, meaning that variations in viral titre prior to development of symptoms cannot be accounted for. In this study, samples were collected shortly before infection, during infection and shortly afterwards, thus allowing for the estimation of viral loads over the entire course of infection. Samples collected in the presence of URTI in this study, had significantly greater RSV viral titres relative to samples collected in the absence of URTI. These results are in agreement with observations in adult challenge studies, which have reported that viral load over the entire course of illness correlates with clinical symptoms (DeVincenzo *et al.*, 2010). These data therefore provide some evidence to suggest that mild RSV infection in infants may be driven by viral load. Evaluation of the association between viral load and different cytokine/chemokine concentrations showed evidence of a positive association between some cytokines/chemokines and viral titre.

Analysis of the kinetics of different cytokine/chemokine responses in the course of natural RSV infection showed that temporal rises in viral load generally corresponded with rises in the levels of certain mediators. The kinetics of the pro-inflammatory response in particular appeared to correlate strongly with virus titre over the course of infection. Peak viral load was in some instances associated with peak concentrations of IL-6, TNF-α and IL-1β as well as presence of URTI. In infants where there was a clear association between virus titre, pro-inflammatory cytokine/chemokine concentration and illness, it can be speculated that illness resulted from both virus mediated necrotic damage of the respiratory epithelia as well as by an increase in the level of pro-inflammatory mediators. This association between rises in the pro-

inflammatory response and illness accords with previous work that has shown that the peripheral production of IL-1β, IL-6 and TNF-α, is linked to activation of neurological pathways that induce physiological changes such as fever and fatigue (Watkins *et al.*, 1995), that are sometimes associated with URTI. For some infants however, although peak cytokine/chemokine levels and peak viral titres coincided with development of URTI, clinical symptoms could not be reliably attributed to RSV infection. For instance in some infants, URTI symptoms were recorded over extended periods that spanned duration of RSV infection, reducing the likelihood that those symptoms occurred exclusively as a result of RSV infection. In some infants, peak viral load and cytokine/chemokine levels occurred in the presence of co-infection with different respiratory pathogens, and therefore confounding the ability to associate symptoms to RSV infection alone.

An interesting association was observed in the kinetic profiles of IFN-γ and IL-10. The dynamics of these cytokines/chemokines appeared to track closely with viral load, despite their clearly antagonistic roles in the host immune response. IFN-γ is a pro-inflammatory Th1 cytokine that has been associated with direct antiviral activity, stimulation of antigen presentation through induction of MHC expression and induction of NK and T cell Cytotoxic activity (Boehm *et al.*, 1997). In the mouse model, RSV infection is dominated by NK cell IFN-γ production, suggesting that this is a key feature of RSV infection *in vivo* (Hussell and Openshaw, 1998, Spender *et al.*, 1998). On the other hand, IL-10 is a potent regulatory molecule, with broad anti-inflammatory properties and plays a role in the regulation of NK cells, T cells and B cells (Moore *et al.*, 2001). Studies in the murine model of RSV have shown that levels of both IFN-γ and IL-10 peak at day 5 post primary infection (Sun *et al.*, 2011)

and that abrogation of IL-10 signalling in this model leads to a substantial increase in disease, manifested as weigh loss (Sun *et al.*, 2011, Loebbermann *et al.*, 2012). The results presented in this chapter are therefore generally consistent with observations in the murine model of co-ordinated IL-10/IFN-γ production at the acute stage of infection. These data provide grounds to suggest that the production of IL-10 in concert with IFN-γ, similarly acts to moderate the effects of host inflammatory responses and thereby limit immune mediated pathology in infants.

An attempt to construct a generic model describing the kinetics of different cytokines/chemokines following natural infection was undertaken by comparing the mean concentrations of different cytokines/chemokines at different time points pre- and post-infection. Mean pre-infection cytokine/chemokine concentrations were compared to cytokine/chemokine concentrations at different time points post infection in order to determine whether natural infection leads to a significant upregulation of certain cytokines/chemokines and also to determine the mean duration of these upregulated responses. In these analyses, only IL-6 and IL-1β were found to be significantly upregulated after infection. The increase in these cytokine/chemokine levels shortly after infection corresponded with an increase in the mean RSV viral load and also corresponded initially with an increase in the proportion of infants who developed URTI. However, later on in the infection, when both viral load and mean IL-6 and IL1-β concentrations were on the decline, the proportion of infants with URTI increased. This suggests that the initial rise in the concentration of these cytokines/chemokines was insufficient to account for the increase symptoms at the initial stages of infection. Unexpectedly, these analyses did not show statistically significant increases in any other cytokine/chemokine following infection. It is likely

that this lack of difference is an analytical artefact arising from the combination of responses from different infants with varying baseline cytokine/chemokine levels.

The effect of virus specific factors on the cytokine/chemokine response to RSV was evaluated by comparing cytokine/chemokine concentrations in the nasal secretions of infants infected with RSV A to those of infants infected with RSV B. With the exception of MCP-4, there was no significant difference between the cytokine/chemokine concentrations produced during RSV A or RSV B infection. The mechanism through which MCP-4 levels were significantly upregulated in the presence of RSV A relative to RSV B was not explored in this study. Despite this differential MCP-4 response, it can be concluded that in general, the two antigenic groups of RSV do not appear to induce broadly variable cytokine/chemokine responses. It has been shown in a previous study that severity of disease as well as viral load does not vary between the two RSV groups (Devincenzo, 2004). The results presented in this chapter suggest that the lack of difference in disease severity following infection with RSV A or B could be related to the failure to induce differential cytokine/chemokine responses.

Chapter 9 - Overall Discussion

Summary of key findings

The work presented in this thesis had the primary aim of describing the role of RSV antigenic variation on the infant serum neutralising response following natural RSV infection. Two secondary goals for this work were to describe the kinetics of the serum neutralising response in infants and to investigate cytokine/chemokine responses in nasal samples obtained from infants with acute RSV infection. The work described in this thesis is the first to comprehensively characterise the group-specificity of the RSV neutralising response in the sera of RSV A infected and RSV B infected infants. A number of studies have previously been undertaken to describe the group-specificity of the infant neutralising response. These studies failed to demonstrate significantly greater homologous responses to both RSV A and B, relative to the respective heterologous responses. For example, Hendry *et al.* (Hendry *et al.*, 1988) and Muelenaer *et al.* (Muelenaer *et al.*, 1991) both sought to describe the group-specificity of the infant serum neutralising response to RSV. However both studies were only able to demonstrate a significantly greater homologous response to RSV B, but failed to show the same relationship in regard to RSV A. On the other hand Roca *et al.* (Roca *et al.*, 2003) showed that infants naturally infected with RSV A generated significantly greater homologous responses against RSV A test strains relative to an RSV B test strain. In addition to the above studies, seminal cross-neutralisation studies carried out in the 1960s failed to demonstrate significant group-specificity to the infant serum neutralising response to both RSV A and B (Coates *et al.*, 1963, Wulff *et al.*, 1964).

The work presented in this thesis is the first to show that the RSV neutralising response to both RSV A and B is significantly group-specific. The work presented further shows that despite 40-50 years of genetic evolution, there was no difference in neutralisation of contemporary and historical strains of both RSV A and B by antibodies generated following natural infection with contemporary virus strains. Further, sera from infants infected with previous strains of RSV neutralised the novel BA variant as effectively as a representative RSV B strain that did not contain the BA duplication.

The duration of the serum neutralising response after primary infection in infants has not been reported. The total serum IgA, IgM and IgG responses to primary infection measured by enzyme immunoassay have been reported to decline to pre-infection levels within one year of infection (Welliver *et al.*, 1980), while the secretory IgA, IgM and IgG responses in infant nasal secretions appear to decline to pre-infection levels by 3 months post infection (Kaul *et al.*, 1981). The temporal dynamics of the neutralising antibody response following primary infection have so far not been reported. The data presented in this thesis show that the infant neutralising response to natural infection is short-lived. Neutralising antibody titres declined to pre-infection levels with 3-4 months post primary infection. Mean neutralising antibody titres in a birth cohort showed mean population level neutralising titres were elevated during epidemics and declined rapidly with a decline in transmission.

Evaluation of cytokine/chemokine responses in nasal samples obtained from infants with acute RSV infection revealed a complex relationship between the kinetics of

cytokine/chemokine concentrations on the one hand and both viral load and disease on the other. There appeared to be some correlation between viral load and the pro-inflammatory response and to a lesser extent the chemokine response. The data showed some evidence of co-ordinated IFN-γ/IL-10 responses in the nasal secretions collected successively from RSV infected infants. In general, there was little evidence of T cell specific responses in the acute stage of mild RSV illness, since, T cell associated cytokines were either undetected or were detected in minimal amounts in infant nasal secretions.

Significance of the findings

Group-specific neutralising antibody immunity

The results presented in this thesis will contribute significantly to the understanding of the transmission dynamics of RSV. In this thesis it was hypothesised that the alternating transmission of RSV A and B is driven by alternation in population level group-specific immunity. The data presented strongly suggest that transient group-specific neutralising antibody immunity at the population level sets the stage for preferential selection for transmission of one group relative to the alternative group. It is likely that following transmission of strains from one group in a particular epidemic, there is a build-up of group-specific neutralising antibody immunity to that group reducing the likelihood of its transmission in the next epidemic. On the other hand as a result of a decline in population level neutralising antibody immunity to the alternative group, there would be a greater likelihood of its transmission in the subsequent epidemic. On the basis of cross neutralisation estimates reported in this thesis the observed alternate transmission patterns of the two groups of RSV in Kilifi Kenya, Turku Finland and in England & Wales have been reproduced in a

mathematical model (White L. Sande CJ *et al.* in preparation). Figure 9.1 is a representation of the model fit, showing that alternating dominance patterns in the different locations can be accounted for by the group-specific immunity estimates obtained in this study. The results presented suggest that vaccines based on only one of the two antigenic groups may provide insufficient coverage against infection with strains from the alternative group. For example, vaccination with a live attenuated vaccine based on an A2 background, may be only partially protective against wild-type group B strains. The implication of this partial protection on population level transmission of RSV may be the continued transmission of strains from the alternative group against a decline in transmission of the vaccine group. This could result in a reduction in the expected indirect protective effect of the vaccine for the vulnerable naïve early infant. To maximize the likelihood of reduced transmission of all RSV strains, it would be beneficial to include representative strains from both RSV A and B in future vaccines.

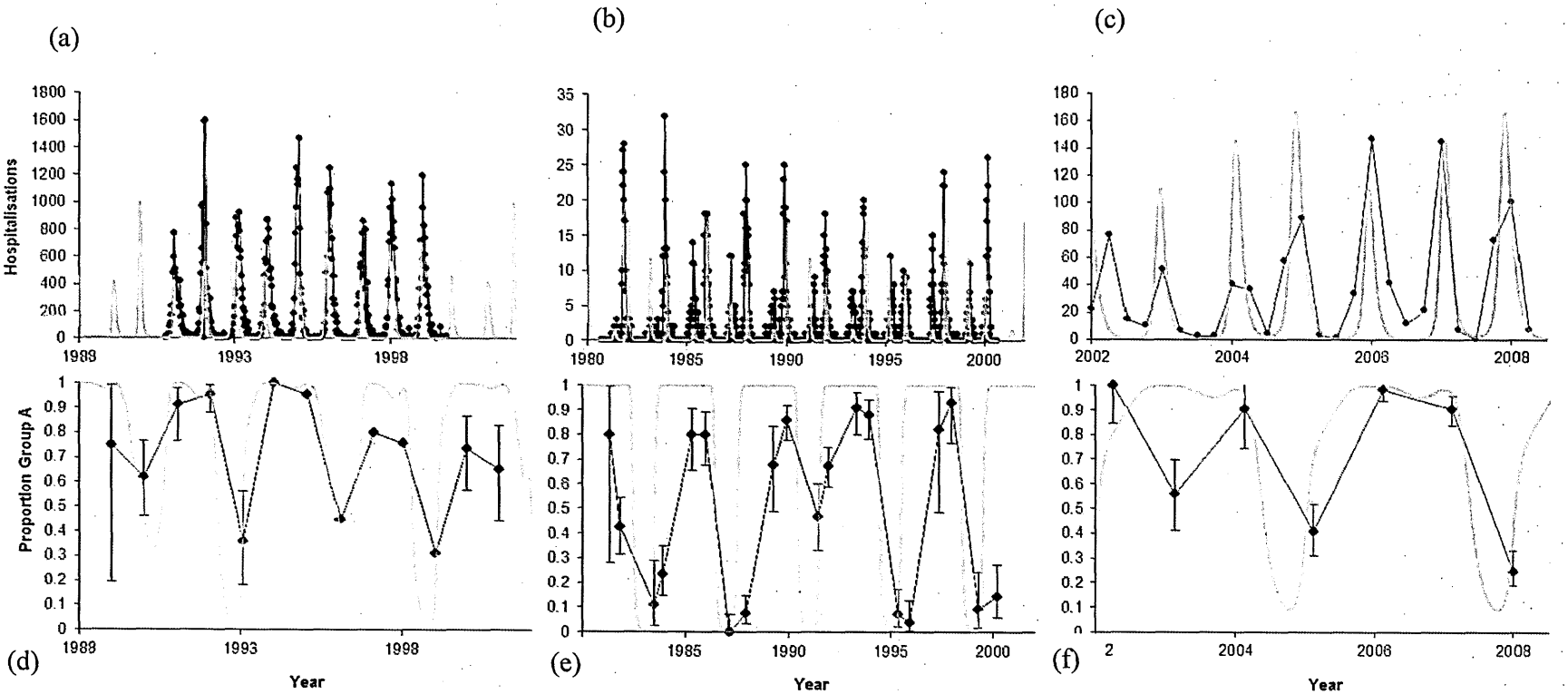


Figure 9.1 Hospitalisations with RSV (top row) and group A dominance dynamics (bottom row) for three geographic locations: England and Wales; Turku, Finland; Kilifi, Kenya. Graphs of the model fit to the time-series data on RSV hospitalizations for three country locations. The weekly hospitalizations (shown as crosses) in England & Wales (a), Turku (b) and (c) Kilifi with the model predictions (solid line). The proportion of typed samples that were group A in England & Wales (d), Turku (e) and Kilifi (f), shown as solid diamonds (with exact binomial 95% confidence interval) and the corresponding predicted proportion from the model (solid line).

Duration of neutralising antibody immunity

The estimates of the duration of neutralising antibody immunity following natural exposure reported in this thesis provide grounds to suggest that the ability of RSV to repeatedly infect is related to short-lived serum neutralising antibodies. The estimates reported further provide the basis upon which an attempt to predict the potential success of future RSV vaccines in reducing the burden of RSV transmission can be undertaken. Many of the viral infections of infancy against which successful vaccines have been developed induce long lived neutralising antibody immunity upon natural infection. On the basis of long-lived protective neutralising antibodies induced by natural infection, it can be argued that the success of vaccination in reducing the transmission burden of these viral infections could have been predicted, assuming that the kinetics of vaccine induced immunity were no different from those of natural infection. For example the introduction of the inactivated polio vaccine in the United States in the mid 1950s and subsequently the oral polio vaccine in the 1960s resulted in a significant and sustained reduction in morbidity and mortality attributed to poliomyelitis (Strebel *et al.*, 1992). Studies done in the late 1940s prior to the introduction of vaccination, demonstrated that natural infection with poliovirus induced neutralising antibody responses that were maintained at protective levels for several decades in the absence of natural re-exposure (Paul and Riordan, 1950). It can therefore be reasonably argued that the success of the poliovirus vaccines could have been anticipated on the basis of the longevity of neutralising antibodies induced by natural infection. Analysis of the duration of acquired neutralising antibodies to RSV show that these antibodies decline to pre-infection levels shortly after natural infection. Further, analysis of mean population level neutralising antibodies in the birth cohort, showed that in the absence of population level virus transmission, mean

population level neutralising titres decline rapidly. These data suggest if future RSV vaccines mediate protection through induction of neutralising antibodies (whose longevity is no different from that produced by natural infection), then it can be assumed that such vaccines are likely to yield only modest reductions in population level virus transmission, since susceptibility to re-infection would be re-established shortly after vaccination. Despite this, such vaccines could still result in significant clinical protection as a result of the priming of immunological memory thereby reducing the potential of subsequent natural challenge resulting in severe illness. These conclusions are based on the assumption that neutralising antibodies are the key correlate of functional immunity. Previous work has shown that protection in animal models can be mediated by antibodies that do not induce *in-vitro* neutralisation of the virus (Plotnicky-Gilquin *et al.*, 1999, Mekseepralard *et al.*, 2006). It is important to note that in the event that antibodies with similar mechanisms of protection play a significant role in human infection, the interpretation of the data presented may be altered by the dynamics of such responses.

The relationship between genetic and antigenic variation of RSV

Genetic studies have produced strong evidence that the attachment protein gene undergoes progressive genetic change with time (Cane and Pringle, 1995, Zlateva *et al.*, 2005, Zlateva *et al.*, 2004). Some studies have shown compelling evidence that suggests that immune selection may underlie the observed changes (Woelk and Holmes, 2001). There has, however, been a lack of data demonstrating the antigenic consequences of these genetic changes, and in particular their effect in mediating the loss of protective antibody responses. The emergence and global distribution of the BA strain of RSV B within a within the last 14 years has brought the role of immune

selection in virus transmission into sharp focus. In this study, no evidence was found to suggest that the BA genetic change resulted in the loss of neutralising ability by serum antibody. Further, evaluation of neutralising responses against virus strains (from the same antigenic group) isolated between 40-50 years apart, showed that temporal genetic evolution was similarly not associated with loss in the neutralising capacity of serum antibody. The data presented suggest that while inter-group genetic differences result in antigenic variation, intra-group genetic differences do not result in antigenic changes that result in differential neutralising responses. There is strong evidence that the infant IgG response to variable parts of the G protein is strain-specific (Cane *et al.*, 1996, Scott *et al.*, 2007), suggesting that the neutralising response to this protein is potentially strain-specific. In this thesis analysis of previously identified neutralising antibody epitopes on the F proteins of infecting wild-type viruses, showed that these epitopes were conserved within or across the group structure of RSV. Weighed against evidence that shows that the F protein contributes more significantly to development of the neutralising response relative to the G protein (Anderson *et al.*, 1988, Olmsted *et al.*, 1986), it is possible that strain-specific neutralising responses targeted at the G protein could have been masked by cross-reactive responses targeted at relatively more conserved epitopes on the F protein. In this study, no attempt was made to study the strain-specificity of neutralising antibodies directed at the G protein exclusive of F-specific responses. It remains to be seen whether in the absence of F-specific neutralising antibodies (removed by absorbing out F-specific antibodies from infant sera), the G-specific neutralising response will be strain-specific. It is also possible that despite the failure to detect genotype-specific RSV serum neutralising antibodies, such antibodies may nonetheless provide protection as suggested by the results of some studies

(Mekseepralard *et al.*, 2006). Therefore while neither the BA genetic change nor temporal evolution was associated with differential *in vitro* neutralising responses, it still remains to be seen whether these genetic variants induce differential *in vivo* protection.

The natural history of the cytokine/chemokine response to RSV

Despite availability of data on the early cytokine/chemokine response in animal models, not much is known on the course of the early cytokine/chemokine response in humans. A key challenge in conducting informative studies in the infant is the inherent limitation in the frequency with which young infants who often have potentially life threatening illness can be sampled. In addition, safety and practical concerns related to sampling of human lung tissue preclude invasive sampling procedures. Due to these concerns, most studies designed to look at the early markers of infant immune response have been based on samples obtained from the upper respiratory tract and presumed to reflect responses in the lower respiratory tract. Many such studies are also conducted at the peak of illness, when infants present to hospital with symptoms of respiratory infection. The key limitation with this approach is that such single time point sampling insufficiently accounts for variations in both viral load and cytokine/chemokine response that may have occurred prior to development of symptoms. In the studies presented in this thesis, infants were sampled at regular intervals during the entire course of infection in order to track the kinetics of both RSV viral load as well as host mediators. These are the first data to describe the infant cytokine/chemokine response to RSV over the entire course of RSV infection and the results reported suggest that RSV viral load drives key cytokine/chemokine responses at the early stages of infection. The pro-inflammatory

response appeared to be particularly strongly linked to viral load while the chemokine response showed moderate correlation with viral load. There was further evidence to suggest that both inflammatory and regulatory responses at the early phases of infection are produced in a co-ordinated fashion in order to modulate the potential pathological effects of unchecked inflammatory responses. Due to limitations in sample volume, no attempt was made in this study to identify the cellular sources of these mediators. While for many infants, peak cytokine/chemokine and viral load responses appeared to be linked with development of URTI, this relationship was not firmly established since in some instances disease was present in the absence of these factors. This unexpected result may be attributable to potential infection by undetected pathogens that resulted in symptoms that were comparable to those produced by RSV infection. Evaluation of virus-specific factors on the cytokine/chemokine response showed that genetic variability between virus strains did not appear to result in differential cytokine/chemokine responses. Most cytokines/chemokines were produced at comparable levels irrespective of whether the infecting strain was RSV A or B.

Limitations of the study and suggestions for future work

The role of G-specific responses on the neutralising response

The work presented in this thesis was based on an *in vitro* neutralisation assay that detected both G- and F-specific neutralising responses. Although previous studies have demonstrated that the anti G-response is strongly genotype-specific (Cane *et al.*, 1996, McGill *et al.*, 2004), in the studies presented in this thesis, a genotype-specific neutralising response was not found. Previous studies have shown that G-specific

antibodies that do not induce *in vitro* neutralisation can confer protection in animal models (Mekseepralard *et al.*, 2006, Plotnicky-Gilquin *et al.*, 1999). In this thesis, the role of strain-specific G protein antibodies in functional protection was not evaluated. Future studies should seek to address the role of antibodies to the G proteins of BA and non-BA strains on *in vitro* protection against experimental challenge with representative BA and non-BA variants.

Group and genotype-specificity of secretory IgA response

The work presented in this thesis, was solely based on serum anti-RSV responses. It can be argued that the key correlate of functional resistance from natural challenge is secretory IgA present on the respiratory mucosa, rather that serum antibody, which is passively transudated to the mucosal surfaces (Wagner *et al.*, 1987a). It is possible that secretory IgA neutralising responses may be slightly different from serum responses in terms of group and genotype-specificity. Future studies should investigate the group and genotype-specificity of the secretory IgA response in order to complement the findings presented in this thesis.

Kinetics of the secondary neutralising response

The ability to detect the temporal dynamics of the neutralising response upon secondary infection was limited by the lack of an adequate number of samples after secondary infection. A comprehensive study of the kinetics of the secondary neutralising response and its relation with the primary response kinetics would advance the understanding of how individuals become resistant to severe infection with age. It is known that most immunocompetent adults have a high titre of anti-RSV

antibodies, relative to infants. The maintenance of these high titres irrespective of recent infection history is likely to be related to resistance against severe disease. Future studies could seek to look at the dynamics of the neutralising response in a birth cohort recruited and followed up for a number of years in order to determine the mechanisms through which older individuals maintain high titres of antibodies. Such studies may help to define antibody titres that are associated with protection from severe disease.

Relative fitness of BA and non-BA strains

Studies on the effect of the BA genetic change on the neutralising response found that this change did not correlate with changes in the neutralising response. This study did not undertake a comparison of the relative fitness of BA and non-BA strains in order to explain variations in transmissibility. Future studies should address the question of whether there is a fitness difference between BA and non-BA strains. This could be achieved by comparing relative replication efficiencies of these strains both *in vivo* (in animal models) and *in vitro* in the presence or absence neutralising antibody. Also, BA and non-BA variants in this thesis were characterised only by limited sequencing of the G gene in order to confirm the presence or absence of the duplication. Since it is possible variations in other parts of the genome could potentially explain the observed variation in transmissibility, future studies should undertake full genome sequencing of these strains in order to determine whether other changes in the genome correlate with differences in viral fitness and therefore differences in transmission.

Relevance of upper respiratory tract sampling to lower respiratory tract disease

Studies on the cytokine/chemokine response to natural RSV infection were conducted on the basis of samples collected from the upper respiratory tract. It is possible that cytokine/chemokine responses in the upper part of the respiratory tract could be different from those in the lower respiratory tract, meaning that conclusions derived from data obtained from upper respiratory tract sampling, would be of limited use in inferring the mechanisms of illness in the lower respiratory tract. A further limitation in this study was the lack of evaluation of the cellular sources of these mediators. In order to definitively characterise these sources it would have been necessary to undertake relatively invasive sampling procedures which are not ethically permissible in infants.

Mathematical modelling of the transmission dynamics of RSV

The group cross-neutralisation estimates obtained in this thesis have been used to model the group replacement dynamics of RSV in three countries (Finland, Kenya and the UK) (White *et al.*, In preparation). The work undertaken so far does not comprehensively address the complex group replacement dynamics seen in other parts of the world, where the pattern of group recurrence is less obvious. The work does not fully address the factors underlying the fact that RSV A is transmitted at a greater frequency relative to RSV B in most parts of the world. Future studies should seek to model the transmission dynamics in countries with diverse population structures, birth rates, and diverse patterns of group recurrence using the estimates provided in this

thesis, in order to further clarify the role of differential immune responses in virus transmission.

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infecting strains of RSV are not antigenically different from those causing primary infection (Beem, 1967), the strain replacement patterns observed in successive RSV epidemics (Cane, 2001), suggests that antigenic variation is an important mechanism through which re-infection occurs. Also recent data shows that a vast majority of re-infecting strains are genetically different from previously infecting strains (Agoti *et al.*, 2012). It may be that natural re-infection is facilitated by both antigenic variation and a short duration of neutralising antibody immunity in young infants. Strain-specific neutralising antibody responses to RSV will be discussed in depth in chapter 7.

The reasons for the short duration of the RSV antibody response following primary infection are not well understood. A number of other viral infections, including paramyxoviruses such as measles and mumps, have been shown to induce antibody responses that are maintained for many years without the need for antigenic stimulation (Amanna *et al.*, 2007). A number of studies have shown the existence of long-lived plasma cells which produce antibody for extended periods without the need for antigenic re-stimulation (Slifka *et al.*, 1998). These data provide the basis to assert that long-lived antibody responses following infection with some pathogens may be the result of long lived plasma cells specific for those pathogens. However a number of studies have also shown than certain plasma cells have a short lived phenotype (Schooley, 1961), suggesting that antibodies produced by these cells are similarly short-lived. Evidence from the murine model suggests that RSV-specific plasma cells after challenge with RSV are not maintained at high levels even after boosting (Singleton *et al.*, 2003). These data suggest that the failure to maintain long-

lived neutralising antibody responses after natural exposure in humans may be related to the short duration of RSV-specific antibody secreting plasma cells.

The memory response to RSV re-infection was explored by looking at the rate of development of neutralising antibodies during primary and secondary infection. Comparison of the mean primary and secondary infection neutralising antibody titres in serum collected within 10 days of identification of virus in nasal samples, showed that while the primary antibody titre was no greater than that of a pre-infection control, it was significantly lower than that of secondary infection. These data suggest that following secondary exposure an immunological memory response is activated and rapidly generates high titres of neutralising antibodies shortly after infection. These data suggest that despite the short duration of primary neutralising antibody responses, protection from severe disease during secondary exposure may be the result of an anamnestic response that acts to rapidly curtail further spread of virus infectivity.

Maternal neutralising antibody protection was analysed by estimating the age prevalence of maternally-derived neutralising antibodies that were above a threshold titre that has been associated with protection from severe disease (Glezen *et al.*, 1981a). The data presented show that there is a rapid decline of protective antibodies shortly after birth. The mean duration of protection of maternally-derived antibodies was found to be of the order of 2.8 months. The proportion of infants who had neutralising antibody titres above this putative protective threshold declined steadily with age and by 3 months of age, none of the infants in this study had maternal neutralising antibodies above this threshold. The results presented have implications

for vaccine design. As previously discussed, there are currently no safe and immunogenic RSV vaccines for use in the early infant population (0-3 months), however live attenuated vaccines that induce protection and that are sufficiently attenuated for older age groups have been reported (Wright *et al.*, 2000). It has been suggested that a realistic vaccination goal under these circumstances might be maternal vaccination, with the ultimate aim of boosting the titre of maternal antibodies that will be eventually transferred into foetal circulation (Englund, 1999). The data presented here suggest that this approach could potentially provide protection in the first few months of life when infants are most susceptible to severe illness. This approach could be of potential benefit among the most vulnerable infant populations such as premature infants - in whom maternally-derived antibody titres are likely to be relatively low at birth. This approach would in such cases boost the titres of maternally-derived antibody and consequently slightly delay the age at which infants first become susceptible to infection and disease.

Chapter 7 - RSV genotype-specific neutralising antibody responses

Introduction

One of the targets of neutralising antibody immunity, the G protein, accumulates genetic changes in the course of viral evolution. This protein has been shown to undergo a rate of molecular evolution of $1.83x10^{-3}$ and $1.95x10^{-3}$ nucleotide substitutions/site/year for RSV A (Zlateva *et al.*, 2004) and B (Zlateva *et al.*, 2005) respectively. Other reports have shown that there is a progressive accumulation of amino acid changes at an average rate of approximately 0.25% per year estimated over the length of the G protein (Cane and Pringle, 1995). This rate of change is comparable to that of the influenza haemagglutinin gene (Cane and Pringle, 1995), which is a main target of protective responses against the influenza virus, and in which temporal molecular evolution correlates with loss of protective immunity (Kilbourne *et al.*, 2002).

The higher rate of non-synonymous to synonymous amino acid substitution observed on the G protein suggests that the changes seen on this protein may be immune driven (Cane and Pringle, 1995). This notion is supported by reports that show the existence of positively selected sites within known antibody epitopes on the G protein (Woelk and Holmes, 2001, Zlateva *et al.*, 2004)). There is indirect evidence that the F protein may also be subject to positive selection pressure, under particular circumstances. Administration of the immunoprophylactic monoclonal antibody Palivizumab, which is specific for the F protein, has been shown to lead to the *in vivo* selection of escape

mutants that contain mutations on the F protein which confer a palivizumab neutralization resistance phenotype (Zhu *et al.*, 2011, Papenburg *et al.*, 2012). *In vitro* studies of 2 palivizumab resistant mutants have shown that one of these mutants rapidly out-competed the prototype A2 genotype in replication competition assays (Zhao *et al.*, 2006), suggesting that immune-selected viruses may be of comparable fitness to other viruses.

Molecular epidemiological studies have produced further indirect evidence that the virus may be subject to positive evolutionary pressure. A number of studies have shown striking similarity between the genotypes of the virus circulating in different parts of world at the same time (Cane *et al.*, 1992). However, these dominant genotypes are often replaced by alternative genotypes in later outbreaks (Choi and Lee, 2000, Seki *et al.*, 2001, Kuroiwa *et al.*, 2005). The molecular evolution of RSV has been characterised as consisting of emergence, decline and sometimes extinction of once dominant genotypes. For example, certain genotypes that were prevalent in the 1970s in Northern Europe but that have not been identified since suggest that they have became extinct (Cane and Pringle, 1995). Viewed together, these data suggest that the genotype structure of RSV is dynamic and is likely to be subject to selection pressures that drive its temporal evolution. Immune pressure is probably key among these selection pressures.

In recent years, a new genotype of RSV B has been reported. This genotype, commonly referred to as the BA genotype, was first isolated in Buenos Aires Argentina in 1998 and has since spread throughout the world and is currently the predominant RSV B genotype in global circulation (Trento *et al.*, 2003, Trento *et al.*,

2010). Its key distinguishing feature is the incorporation of a 60 nucleotide duplication in the variable part of the G protein (Trento *et al.*, 2003). The nature of this mutation as well as the fact that it had not hitherto been identified in any previous circulating genotype, suggests that it is unlikely to have arisen from more than one infection event in a single individual and accordingly the dynamics of its global spread can be used as a model of the transmission dynamics of other genotypes of RSV. The remarkable transmission success of BA strains relative to previous RSV B genotypes can be hypothesized to have been the result of an immune selection advantage that conferred upon it the ability to out-compete other group B genotypes in a natural transmission setting. To date there have been no studies published that explore this phenomenon. In 2012, a wild-type variant of RSV A was identified which incorporates a 72 nucleotide duplication in the variable part of the G gene, reminiscent of the BA genotype (Eshaghi *et al.*, 2012). It is yet to be seen whether this new variant will achieve comparable epidemiological success to the BA genotype.

This chapter will explore the effect of genotype-specific variation in RSV on the neutralising antibody response to natural RSV infection as well the effect of the BA genetic change on the neutralising responses of infants infected with different RSV B genotypes

Chapter Aims

This chapter will describe the genotype-specificity of the RSV neutralising response. Responses to two strains of RSV A and two strains of RSV B will be evaluated in the serum of infants infected with wild-type genotypes of RSV A and B. The genetic characteristics of both infecting and test genotypes will be related to genotype homologous and heterologous neutralising antibody responses. The results will be

discussed in terms of the effect of molecular evolution on the neutralising response and its implications on the development of live attenuated vaccines. The chapter will also evaluate infant serum responses to group conserved regions on the attachment protein. These results will be discussed in the context of development of bivalent live attenuated vaccines comprising representative genotypes from the two RSV groups.

Materials and methods

Study population, sampling and molecular characterisation of test and infecting viruses

Nasal washings were obtained from children less than 60 months of age admitted to KDH with RSV-associated severe or very severe pneumonia. Laboratory diagnostic techniques used in this study are the same as those described in chapter 5. The test viruses used in these studies were: the A2 (RSV A; isolated in Australia in 1961), Kil/A/2006 (RSV A; Kenya, 2006), 8/60 (RSV B; Sweden, 1960) and Kil/B/2008 (RSV B; Kenya, 2008). The attachment and fusion protein genes of all the test viruses as well as a number of infecting strains were sequenced as described in Chapter 3. The neutralisation assays used in this study are described in chapter 4. Complement specific neutralisation assays were conducted by addition of 10% guinea pig complement (Sigma) to infant sera. Comparison of nucleotide and amino acid sequence identity was done using Openoffice.org calc software (Apache software foundation, Forest Hill, Maryland USA).

Enzyme Linked Immunosorbent Assays (ELISA) using synthetic peptides

Infant serum IgG responses to conserved regions on the central portion of the G genes of RSV A and B were measured using ELISA. The synthetic peptides spanned amino acid 170-189 of the RSV attachment protein. The RSV A constant region comprised the following amino acids: FVPCSICSNNPTCWAICKRI while the RSV B constant region comprised the following amino acids: FVPCSICGNNQLCKSICKTI. Synthetic peptides matching these sequences were diluted in dimethyl sulphoxide (DMSO) to a working concentration of 10 μM. A checkerboard titration assay was carried out using pooled convalescent sera to determine the optimal serum and synthetic peptide concentrations for the assays. The results of this assay are shown in Figure 7.1. On the basis of these results it was decided that a serum dilution of 1:100 and a synthetic peptide concentration of $3.1x10^{-6}$ M were optimal for the assay. The ELISA was carried by first coating 96 well polystyrene plates (Nunc MaxiSorp, Nunc Corporation) overnight at 37°C in a humidified CO_2 incubator with the appropriate concentration of peptide diluted in phosphate buffered saline. Plates were then blocked for an hour, using a blocking buffer consisting of 5% powdered milk (Marvel) in PBS. 100 μl of infant sera diluted 1:100 in the blocking buffer, was then added to the wells of the 96 well plates and incubated for one and a half hours at 37°C. The plates were then washed 3 times in PBS containing 0.05% Tween 20, followed by addition of 100 μl per well of a 1:1000 dilution of horseradish peroxidase (HRP) tagged rabbit anti human IgG (Dako corporation) diluted in blocking buffer. After incubation for 1 hour at 37°C, the plates were washed 3 times as before and developed. The development solution was prepared by adding 10 μg orthophenylenediamine (OPD) tablets into 10 ml of PBS and aliquoting 100 μl of this solution to the appropriate wells of the 96 well reaction plate. This reaction was

incubated for 10 minutes in the dark and stopped by the addition of 50 µl of 2.5 M H_2SO_4 into the appropriate wells of a 96 well plate. The plates were then read on a standard microplate reader at 495nm.

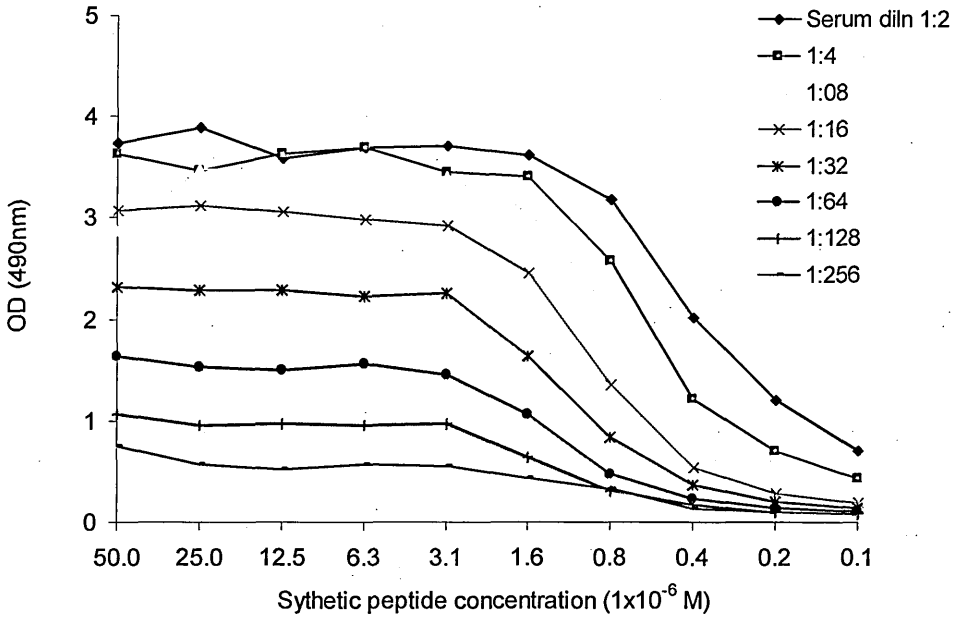

Figure 7.1 The results of a checkerboard titration assay in which varying dilutions of sera were titrated against decreasing concentrations of synthetic peptides in order to determine the optimum concentration of either parameter.

Data Analysis

Inference of the genetic relationship between different infecting and test genotypes was carried out using Multi Dimensional Scaling (MDS) and phylogenetic analysis. Pairwise distance matrices containing the level of variation between different sequences in terms of nucleotide substitutions per site were generated using MEGA software (Build 5110426) using the maximum composite likelihood model. These distance matrices were then subjected to classical MDS analysis using Matlab (release R2008b, MathWorks Inc). MDS analysis resulted in the generation of two dimensional plots graphically depicting the genetic relationship between different

genotypes based on their relative pairwise distances from each other. The genetic relationship between different genotypes was also assessed by comparing the level of amino acid conservation by identification of conserved amino acid residues as well as by the use of phylogenetic techniques. Phylogenetic analysis was carried out by generation of maximum likelihood phylogenetic trees consisting of G gene sequences from different genotypes.

Comparison of the strain homologous and heterologous responses was done by analysing differences in fold rise in titre to homologous and heterologous virus as well as by differences in the ability to seroconvert. A multiple regression model was used to determine whether the magnitude of the neutralising response to the homologous virus was statistically different from that to the heterologous response. McNemar's chi square test was used to compare the proportions seroconverting to homologous or heterologous virus. Responses to group conserved synthetic peptides in acute and convalescent phase sera in terms of optical density changes were compared using paired student t tests. The analytical output for the data presented in this chapter is presented in appendix 3.

Results

Comparison of sequence data between different genotypes of RSV B

Comparison of F and G gene amino acid sequences of infecting BA (n=20) and non-BA (n=20) strains was done in terms of nucleotide and amino acid identity. There was 95.1% and 97.1 % nucleotide and amino acid identity, respectively, shared between F proteins of all RSV B strains in the study, irrespective of whether they

were BA or non-BA. On the other hand there was 79.7% and 73.8% nucleotide and amino acid identity, respectively, shared by the G proteins of all infecting RSV B strains in the study. Analysis of the BA strains alone showed that they shared 98% and 98.6% nucleotide and amino acid identity respectively at the F gene level and 95.2% and 93.8% nucleotide and amino acid identity, respectively, at the G gene level. Analysis of the non-BA strains showed that they shared 99.5% and 98.6% nucleotide and amino acid sequence identity, respectively, at the F protein level and 97.6% and 94.3% nucleotide and amino acid sequence identity, respectively, on the G protein. These data are presented in tabular form in Table 7.1. Analysis of the variation between the 2 RSV B test strains, Ken/B/2008 (BA) and Swe/B/1960 (non-BA) showed that they shared 77% amino acid identity on the G protein.

Comparison groups (n)	Amino acid identity (%)		Nucleotide identity (%)	
	F protein	G protein	F gene	G gene
RSV B only (BA & non-BA) (40)	97.1	73.8	95.1	79.7
BA only (20)	98.6	93.8	98	95.2
Non-BA only (20)	98.6	94.3	99.5	97.6

Table 7.1 Analysis of the level of nucleotide and amino acid identity among different genotypes of RSV A and B

Analysis of level of genetic conservation on some neutralising antibody epitopes on the F and G proteins of different RSV B genotypes

The level of amino acid conservation on selected neutralising epitopes was compared between BA and non-BA strains of RSV B. The F protein epitopes that were analysed

were obtained from published literature and are illustrated in figure 5.12 in Chapter 5. The four neutralising epitopes were located between the following amino acid positions on the primary sequence of the F protein: (i) amino acids 205-225 (Bourgeois *et al.*, 1991), (ii) amino acids 221-236 (Trudel *et al.*, 1987b), (iii) amino acids 262-268 (Lopez *et al.*, 1990) and (iv) amino acids 289-298 (Martin-Gallardo *et al.*, 1991). All the neutralising epitopes on the F protein were found to be perfectly conserved on wild-type BA and non-BA strains in this study (shown in Figure 5.12).

Analysis of the level of amino acid conservation on the G proteins of infecting BA and non-BA strains was carried out next. Two neutralising epitopes that occurred between amino acids 151 - 172 (Murata *et al.*, 2010) and amino acids 201 - 213 (Garcia-Barreno *et al.*, 1992) were evaluated. There were two genotype-specific amino acid differences between wild-type BA and non-BA viruses on the epitope spanning amino acids 151 and 172, while there was only one amino acid difference in the epitope spanning amino acids 201 and 213. There were, however intra-genotype variations identified on this epitope. One of the BA sequences varied from other BA sequences by containing a Proline to Leucine substitution at position 207. Also, two other BA sequences varied from all other group B sequences since they contained a Leucine to Isoleucine substitution at position 201 of this epitope. These differences are illustrated in figure 5.12.

Analysis of the genetic and antigenic relationship between infecting RSV B genotypes

Inference of the genetic relationship between the G genes of BA and non-BA genotypes was carried out using both Multi Dimensional Scaling (MDS) and

phylogenetic analysis. Results of MDS analysis showed that G gene sequences obtained from infants infected with wild-type BA strains clustered together on the two dimensional MDS space, while those obtained from infants infected with non-BA genotypes, similarly clustered together, but on a separate region of the two dimensional space. For comparison, both the Swe/B/1960 and Ken/B/2008 test genotypes were included in the MDS analysis. The Swe/B/1960 genotype was separated from both the BA and non-BA clusters by a considerable genetic distance while the Ken/B/2008 test genotype was located within the cluster of wild-type BA sequences. The genetic relationship between BA, non-BA, Swe/B/1960 and Ken/B/2008 on the two dimensional MDS space is shown in figure 7.2.

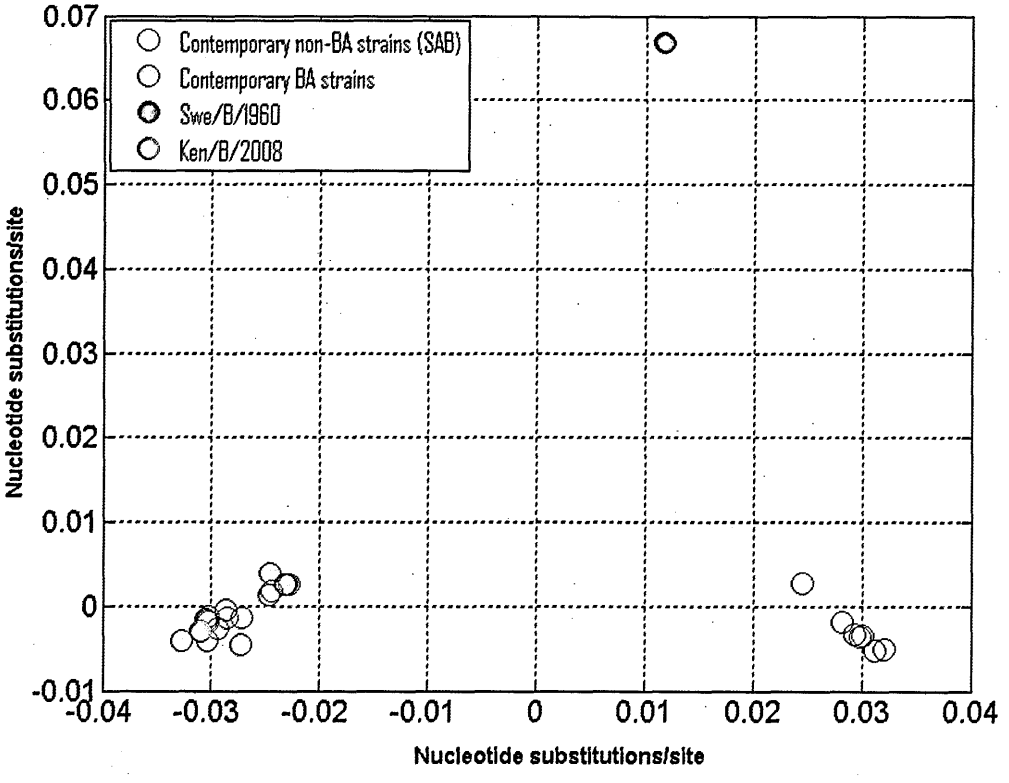

Figure 7.2 The results of MDS analysis in which the relationship between the G genes of different infecting (20 BA viruses and 20 non-BA viruses) and test strains of RSV B is depicted on a two dimensional space. Each spot represents one sequence and their co-ordinates on the cartesian plane are based on their relative pairwise distances. Identical sequences are represented by a single point on the Cartesian plane. The x and y axes

represent pairwise distances in terms of number of nucleotide substitutions per nucleotide site between different sequences.

For comparison, a maximum likelihood phylogenetic tree showing the genetic relationship between different RSV B genotypes was constructed with 100 bootstrap replications. Results of the phylogenetic analysis showed that wild-type BA and non-BA genotypes were located on separate branches of the phylogenetic tree while the Swe/B/1960 genotype was distantly separated from both BA and non-BA infecting virus clusters. On the other hand, the Ken/B/2008 genotype was located within the branch on which wild-type BA genotypes were located. The phylogenetic tree is graphically presented in figure 7.3.

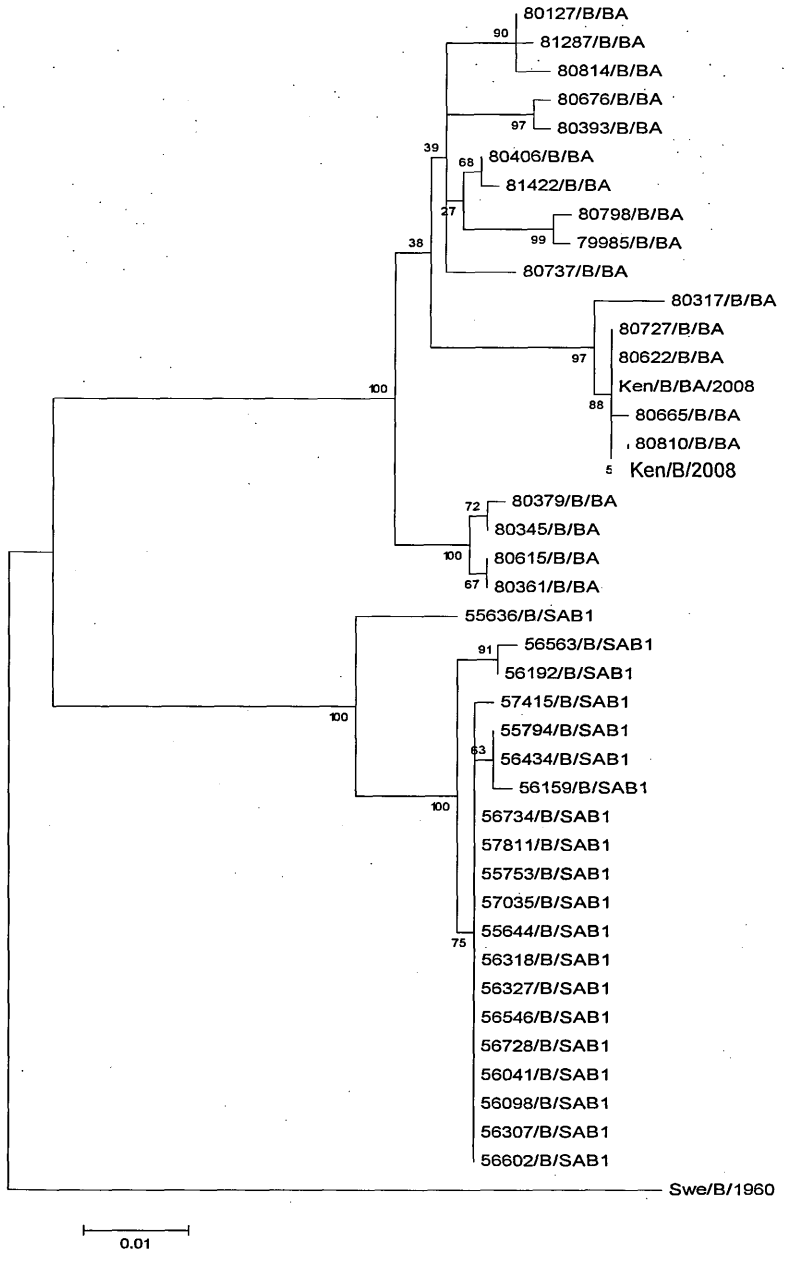

Figure 7.3 The phylogenetic relationship of partial G gene sequences (between nucleotide 284 of the G gene and nucleotide 9 on the F gene) obtained from both infecting and test viruses are shown in this maximum likelihood tree. The notational convention for the wild-type genotypes is as follows: patient ID/Infecting group/RSV B genotype. Notation for test genotypes follows the following order: Place of isolation (Swe=Sweden; Ken=Kenya)/Infecting group/Year of isolation.

To assess whether the genetic differences between BA and non-BA genotypes were reflected in terms of differential neutralisation, inference of the antigenic relationship between the infecting BA and non-BA genotypes was done using classical MDS. This

analysis utilized cross neutralisation data obtained from genotype homologous and heterologous reactivity. The test viruses in these experiments were 8/60 (non-BA) and Ken/B/2008 (BA). Titres to either test strain were measured using the plaque reduction neutralisation assay. Neutralising responses to homologous or heterologous virus were measured in terms of fold rise in titre from the acute to convalescent phases of infection. The results of these analyses, shown in figure 7.4, show no evidence of differential clustering of antibody responses to the BA or non-BA genotypes. Stronger homologous to heterologous to either test genotype neutralisation would have resulted in separate clustering of BA and non-BA specific responses on the 2 dimensional MDS space, indicating differential patterns of recognition of the two genotypes.

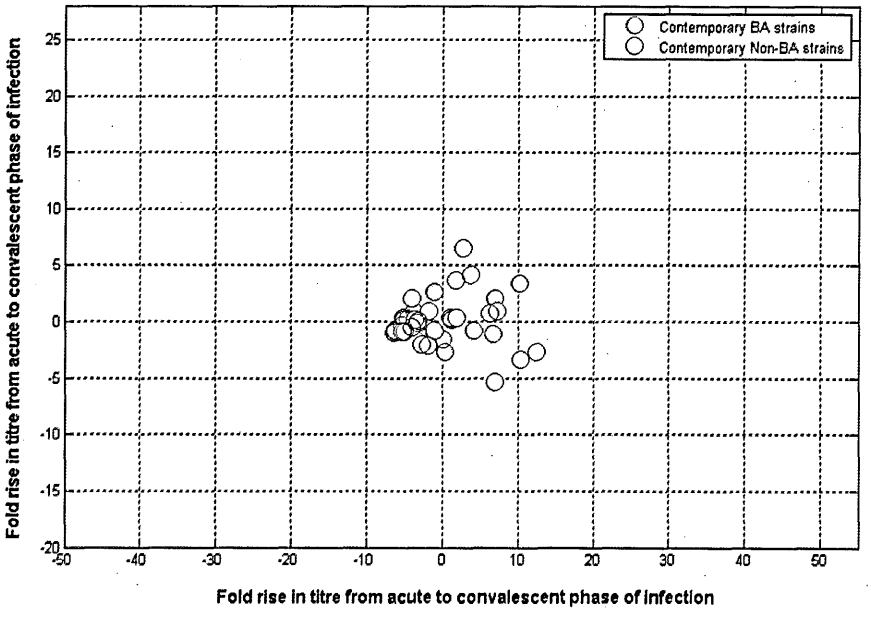

Figure 7.4 Multidimensional scaling (MDS) plot showing the antigenic relationship between BA and non-BA strains of RSV B. Sera from infants infected with BA (n=20) or non-BA (n=20) viruses were used to neutralise representative non-BA (8/60 virus) and BA (Ken/B/2008 virus) strains. The resulting cross neutralisation matrix data (expressed as fold rise in titre from the acute to convalescent phase of infection) was used to construct a two dimensional MDS plot depicting the antigenic relationship between BA and non BA strains of RSV B.

Statistical Analysis of homologous and heterologous neutralising responses to BA and non-BA genotypes of RSV B

Homologous and heterologous neutralising responses to representative BA (Kil/B/2008) and non-BA (Swe/B/1960) test strains were next compared for evidence of statistically significant differences. There was no significant difference between the magnitude of the neutralising response mounted by infants infected with non-BA strains (n=20) to the Swe/B/1960 virus (3.7 fold rise in titre) and the Kil/B/2008 virus (3.42-fold rise in titre; p=0.78). There was also no significant difference between the magnitude of the neutralising response mounted by infants infected with BA strains (n=20) to the Swe/B/1960 virus (5.13 fold rise in titre) and the Kil/B/2008 virus (3.54-fold rise in titre; p=0.1). These data are presented graphically in figure 7.5.

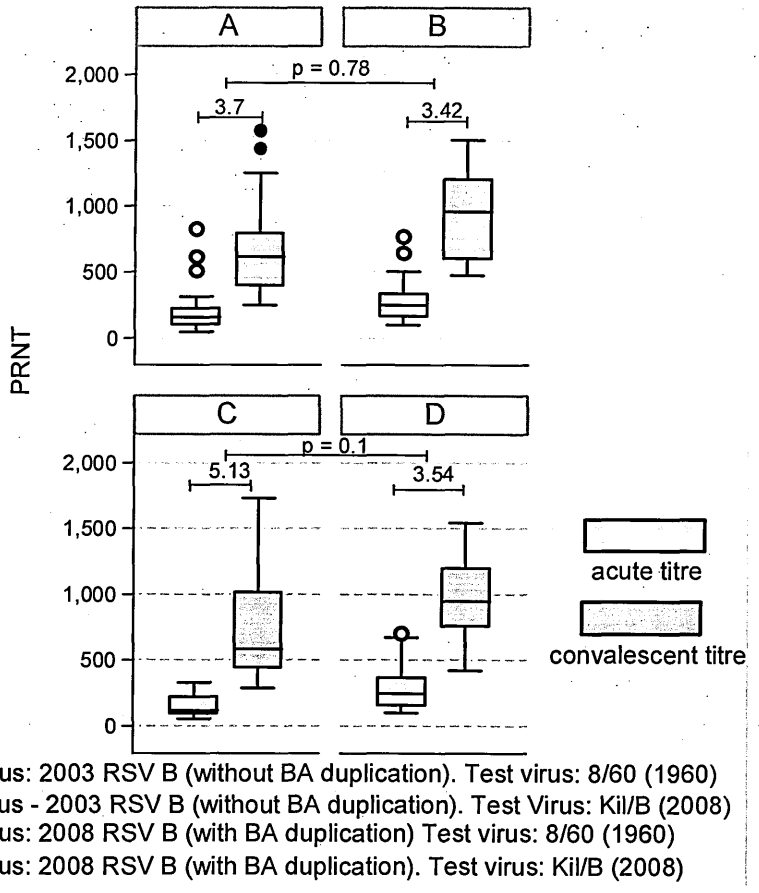

Figure 7.5 Acute and convalescent phase responses to representative BA (Ken/B/2008) and non-BA (8/60) test viruses among infants naturally infected with wild-type BA (n=20) and non-BA (n=20) viruses. The numbers above each acute/convalescent serum pair denote the fold rise in titre from the acute to convalescent phases of infection. The p value bars traversing the panels indicate the level of statistical significance between genotype homologous and heterologous responses.

Serum neutralising responses from infants infected with BA or non-BA strains were compared in terms of their ability to seroconvert to the Kil/B/2008 and Swe/B/1960 test viruses. Out of 20 individuals infected with wild-type non-BA strains, the proportion that seroconverted to the BA test virus Kil/B/2008 (35%) was not statistically different from the proportion that seroconverted to the non-BA test virus, Swe/B/1960 (50%; McNemar's χ^2=1.8, p=0.4). Among the 20 individuals infected with wild-type BA strains in the study there was no difference between the proportion

that seroconverted to the BA test virus (50%) and the proportion that seroconverted to the non-BA test virus (65%; McNemar's χ^2=1.8, p=0.4).

The effect of complement on genotype-specific neutralisation of the group B genotypes was evaluated using sera from 20 infants; 10 who had wild-type infections with BA strains and 10 who had wild-type infections with non-BA strains. Seroconversion rates to both the Kil/B/2008 virus and the Swe/B/1960 virus were compared in the presence and absence of complement. The proportion of infants infected with non-BA genotypes and who seroconverted to the Swe/B/1960 virus in the presence of complement (40%) was no different from the proportion that seroconverted in its absence (40%, McNemar's χ^2=1, p=1). The proportion of infants infected with BA strains in the 2007/2008 epidemic who seroconverted to the Swe/B/1960 virus was also no different in the presence (50%) or absence of complement (60%, McNemar's χ^2=1, p=0.16). There was similarly no difference in the proportion of infants infected with non-BA strains who seroconverted to the Kil/B/2008 virus in the presence (40%) or absence of complement (30%, McNemar's χ^2=1, p=0.3173). Finally, the proportion of infants infected with BA strains that seroconverted to the Kil/B/2008 virus in the presence of complement (50%) was similar to the proportion that seroconverted in its absence (30%, McNemar's χ^2=1, p=0.3173).

Neutralising responses to contemporary and historical genotypes of RSV A

The relationship between 20 infecting RSV A viruses was analysed by identifying conserved nucleotide and amino acid residues along the length of the fusion and

attachment proteins. There was 96.8% and 98.1% nucleotide and amino acid identity respectively on the F proteins of infecting RSV A viruses. On the other hand, there was 94.8% and 91.4% nucleotide and amino acid identity respectively on the G proteins of infecting viruses. These data are shown in table 7.2. Analysis of the level of amino acid conservation on some neutralising antibody epitopes on F and G protein of infecting RSV A viruses (shown on figure 5.12 and figure 5.13) showed that all neutralising antibody epitopes tested on both proteins were perfectly conserved on all infecting RSV A viruses sequenced. Comparison of the level of genetic variation between the 2 group A test viruses, Ken/A/2006 and A2 showed that they shared 88% amino acid identity on the G protein.

Comparison groups (n)	Amino acid identity (%)		Nucleotide identity (%)	
	F protein	G protein	F gene	G gene
RSV A only (20)	98.1	91.4	96.8	94.8

Table 7.2 Comparison of genetic relatedness of infecting RSV A genotypes in terms of percent nucleotide and amino acid identity

The genetic relationship between the G genes of RSV A viruses was further inferred using MDS. Pairwise distances between the G genes of infecting RSV A viruses were generated and plotted on a two dimensional MDS plot. The results of these analyses are shown in figure 7.6. The results show a high level of clustering of infecting RSV A G genes. Due to the high level of genetic conservation in the G protein of infecting RSV A viruses, many viruses were located on the same co-ordinates on the two dimensional MDS plot shown in Figure 7.6. The Ken/A/2006 test virus was located within the cluster of contemporary RSV A infecting viruses, while the A2 test genotype was distantly separated from this cluster.

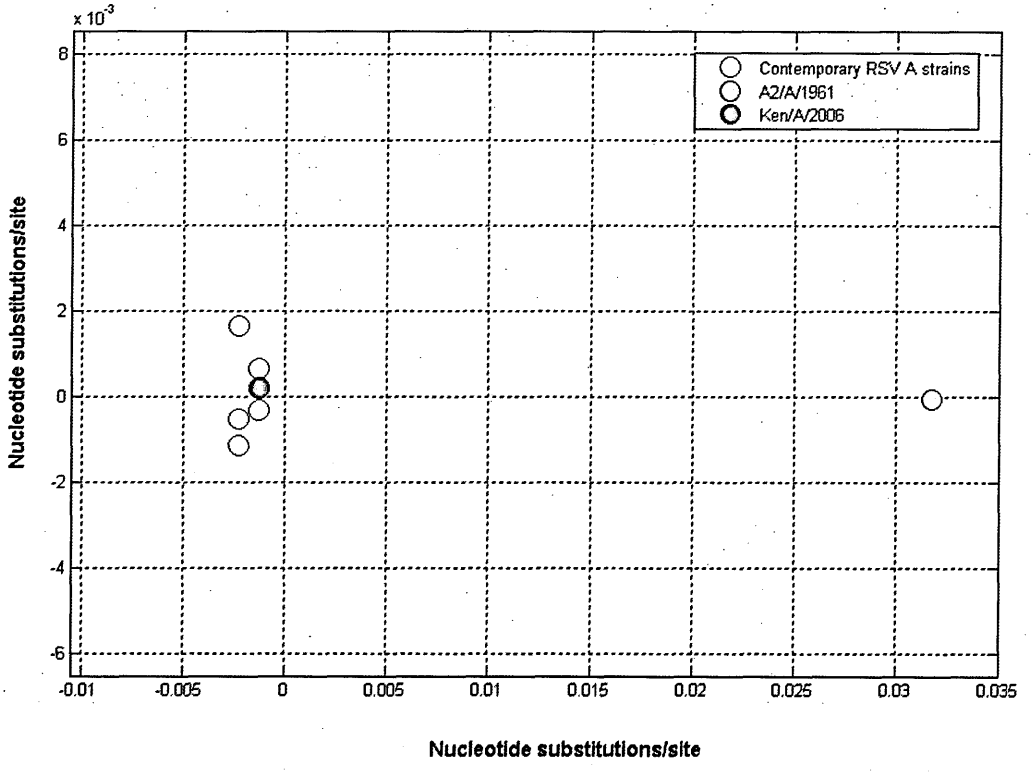

Figure 7.6 Multidimensional scaling plot showing the genetic relationship between the G genes of 20 infecting RSV A strains and the two RSV A test viruses. Closely related or identical sequences are represented by overlapping spots on the two dimensional plot. The unit of measurement on the x and y axes is number of nucleotide substitutions per site.

The genetic relationship between the infecting and test viruses of RSV A was confirmed using phylogenetic analysis. A maximum likelihood tree with 100 bootstrap repetitions was constructed using MEGA software. The results of these analyses which are shown in Figure 7.7 show that there was a close genetic relationship between all infecting RSV A viruses. However, the A2 test virus was found to be distantly related to the infecting RSV A viruses.

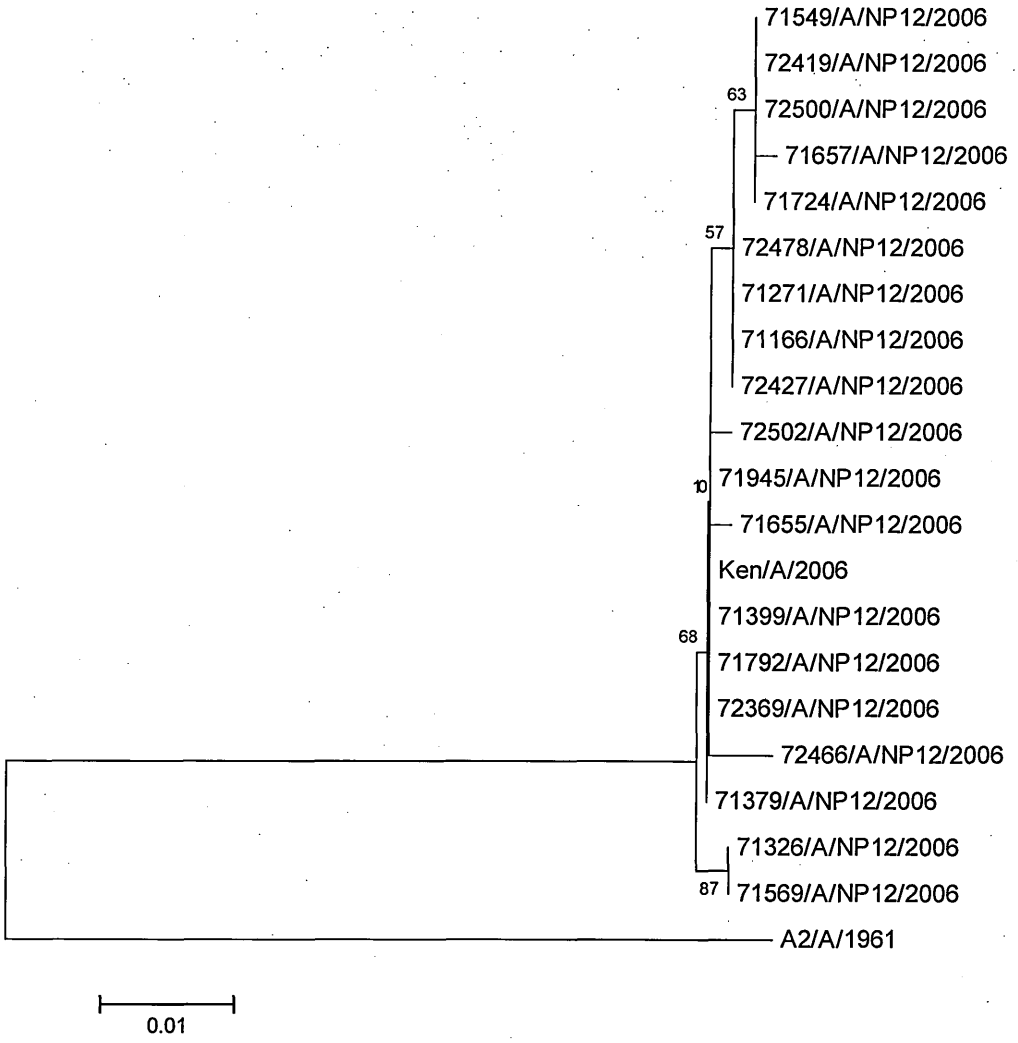

Figure 7.7 Maximum likelihood phylogenetic tree showing the relationship between the G genes of infecting and test RSV A viruses. The notational convention for the wild-type genotypes is as follows: patient ID/Infecting group/NP genotype/year of isolation. Ken/A/2006 refers to the contemporary RSV A test strain while A2/A/1961 refers to the historical A2 strain

Comparison of the neutralising antibody response by infants infected with contemporary RSV A genotypes to both the A2 (Aus/A/1961) and Kil/A/2006 test viruses.

Statistical comparison of genotype-specific neutralising responses in the sera of infants infected with contemporary RSV A viruses to the contemporary and historical RSV A test viruses was carried out next. There was a mean 3.42-fold rise in titre against the A2 test virus and a mean 3.15-fold rise in titre to the Kil/A/2006 test virus. Results of regression analysis showed that the difference in these responses was not statistically significant (p=0.8). This comparison is graphically depicted in Figure 7.8.

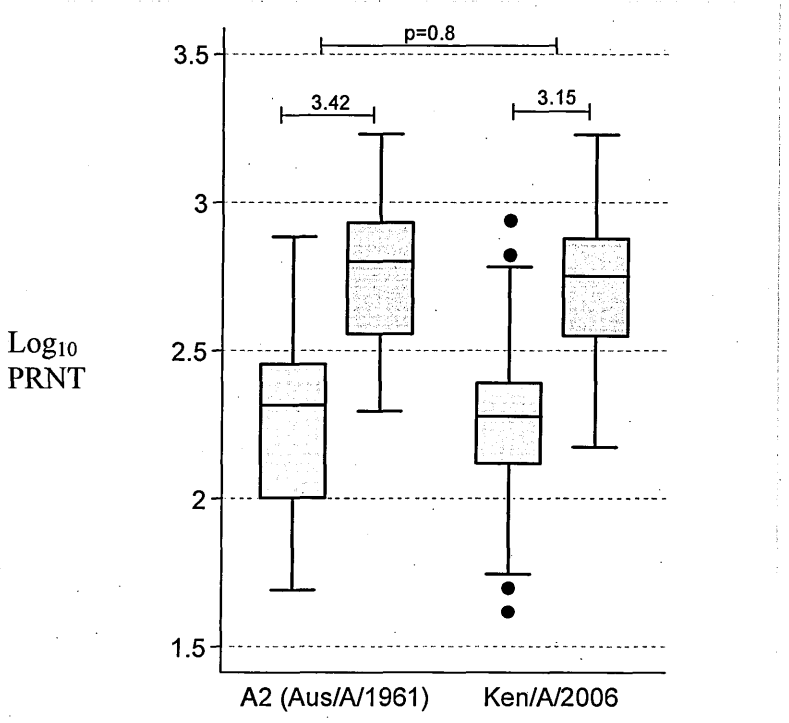

Figure 7.8 Comparison of the magnitude of the neutralising response to the A2 and Ken/A/2006 test viruses of RSV A. The light grey bars represent acute stage titres while the dark grey bars denote convalescent phase responses. The numbers shown above each acute/convalescent sera pair denotes the fold rise in titre from the acute to convalescent phases of infection. The p-value shows that the magnitude of the neutralising response to these two viruses was not significantly different.

Responses to the A2 and Kil/A/2006 viruses were also evaluated in terms of ability to seroconvert. Of the 33 RSV A infected individuals tested against both A2 and Kil/A/2006, no significant difference was found between the proportion that seroconverted to the A2 virus (39.4%) and the proportion that seroconverted to the Kil/A/2006 virus (51.5%; McNemar's χ^2=4, p=0.13).

Responses to group conserved regions on the attachment protein

Antibody responses to synthetic peptides matching group conserved regions on the central part of the G molecule were evaluated in the sera of infants with RSV A (n=57) and B (=34) infections who had been admitted with severe RSV-associated pneumonia. Acute and convalescent phase titres from the sera of infants admitted with severe RSV were compared using a paired two sided student's t-test. Comparison of responses to the RSV A constant region showed that the convalescent response in the sera of RSV A infected infants was significantly greater than their acute phase response (t=-6.4, p<0.0001). On the other hand, there was no difference between the acute and convalescent phase responses in the sera of RSV B infected infants to the constant region of RSV A (t=-0.13, p=0.9). These data are shown graphically in figure 7.9.

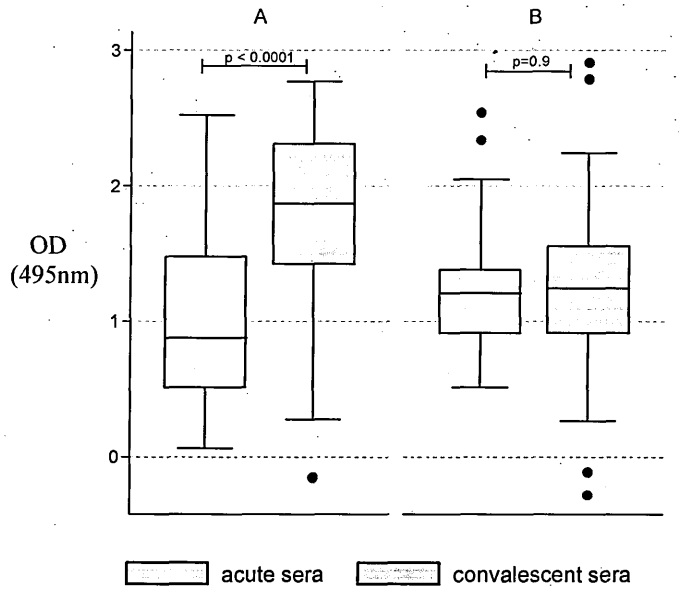

Figure 7.9 Comparison of responses to the conserved region of the RSV A G gene in the sera of infants with natural RSV A (left panel) or RSV B (right panel) infections

This homotypic response pattern was repeated in the infant response to the constant region of RSV B infected individuals. While there was no statistically significant difference between the acute and convalescent phase responses in the sera of RSV A infected individuals against the RSV B constant region ($t=-0.6$, $p=0.6$), the convalescent phase response of RSV B infected infants to the RSV B constant region was significantly greater than the acute phase response ($t=-10.3$, $p<0.0001$). These responses are graphically represented in figure 7.10.

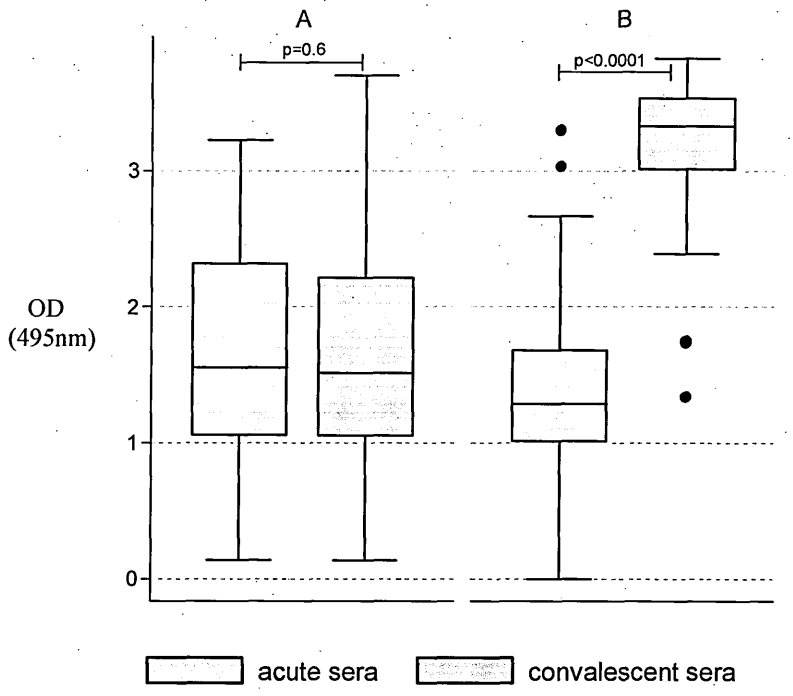

Figure 7.10 Comparison of responses to the conserved region of the RSV B G gene in the sera of infants with natural RSV A (left panel) or RSV B (right panel) infections

Responses to the RSV A and B constant region were further evaluated in terms of seroconversion. Of the 16 infants that seroconverted to the RSV A constant region, 16 (100%) were infected with RSV A while none (0%) were infected with RSV B. This difference was statistically significant (χ^2=11.6, p=0.001). Similarly of the 7 individuals who seroconverted to the RSV B constant region, 7 (100%) were infected with RSV B while none (0%) were infected with RSV A. This difference was also statistically significant (χ^2=12.7, p<0.0001). These data are shown in table 7.3 and table 7.4.

Sero-conversion	Infecting group A n (%)	B n (%)	Total n (%)
No	41 (55)	34 (45)	75 (100)
Yes	16 (100)	0 (0)	16 (100)
Total	57 (63)	34 (37)	91 (100)

Table 7.3 Comparison of the proportion seroconverting to the RSV A constant region in the sera of RSV A or RSV B infected infants

Sero-conversion	**Infecting group A** n (%)	**B** n (%)	**Total n (%)**
No	57 (68)	27 (32)	**84 (100)**
Yes	0 (0)	7 (100)	**7 (100)**
Total	**57 (63)**	**34 (37)**	**91 (100)**

Table 7.4 Comparison of the proportion seroconverting to the RSV B constant region in the sera of RSV A or RSV B infected infants

Discussion

The first part of the studies presented in this chapter looked at responses to two representative RSV B test viruses among infants who had experienced natural infection with wild-type BA or non-BA strains. One of the two representative test viruses (Kil/B/2008 – isolated in Kilifi in 2008) contained the 60 nucleotide duplication while the other (Swe/B/1960 – isolated in Sweden in 1960) did not.

Direct comparison of the infecting F and G gene sequences showed there was a high degree of relatedness in terms of nucleotide and amino acid identity. The F gene was the most highly conserved molecule with over 90% nucleotide and amino acid identity within and between the two infecting RSV B genotypes. The G protein was less conserved with 73.8% and 79.7% amino acid and nucleotide sequence identity respectively shared between infecting BA and non-BA genotypes of RSV B. Analysis of four neutralising epitopes on the F proteins of infecting viruses showed that they were perfectly conserved both within and between the two infecting group B genotypes. Since the F protein, is the major target of neutralising antibody responses (Olmsted *et al.*, 1986, Anderson *et al.*, 1988), these data strongly suggested that neutralising responses directed at one genotype were likely to be effective against the alternative genotype. Extension of these analyses to neutralising epitopes on the G protein found evidence of genotype-specific amino acid differences within neutralising epitopes. Of the two G protein epitopes evaluated, the first, spanning amino acids 151 and 172, contained two genotype-specific amino acid differences, while the other, which spanned amino acids 201 and 213, contained one genotype-specific amino acid difference.

Analysis of the genetic relatedness of infecting BA and non-BA genotypes carried out by Multidimensional Scaling (MDS), showed evidence of inter-genotype genetic variation but little evidence of intra-genotype variation. In general, infecting BA viruses tended to cluster closely together on the two dimensional MDS space while non-BA viruses clustered together in a similar manner to BA viruses, but were separated from the BA cluster by a considerable genetic distance. While the BA test virus (Ken/B/2008) was located within the cluster of infecting BA viruses, the non-

BA test virus (Swe/B/1960) was located distantly from both infecting virus clusters. This suggests that the Swe/B/1960 virus has undergone considerable genetic evolution relative to the contemporary BA and non-BA infecting genotypes. Non-BA viruses in this study were isolated in the 2002/2003 RSV epidemic in Kilifi while the BA viruses were collected in the 2007/2008 epidemic, meaning that between 43 and 48 years respectively of temporal evolution separated the Swe/B/1960 test virus from the contemporary group B infecting viruses. This relationship was confirmed by phylogenetic analysis, which showed differential clustering of BA and non-BA infecting viruses and marked separation of the Swe/B/1960 genotypes from both clusters.

The effect of these genetic differences on the serum neutralising antibody response was evaluated by measuring neutralising responses in the acute and convalescent sera of infants infected with either genotype to both test viruses. Analysis of the serum neutralising responses of infants naturally infected by either wild-type BA or non-BA viruses, showed that their respective responses both in terms of fold rise in titre from the acute to convalescent phases of infection as well as their ability to seroconvert to either test virus was not statistically different. MDS analysis confirmed that the neutralising response of infants infected with BA viruses was no different from those of infants infected with non-BA viruses, since there was no evidence of differential clustering of antibody response in the two dimensional MDS space.

The data showing strong genetic variation on the G genes of contemporary and historical strains support previous work that showed the existence of strong molecular evolution acting on the G protein. There was indirect support for the idea that the

observed changes on the G protein could have been driven by immune pressure since a number of amino acid changes were identified on neutralising antibody epitopes located on the G protein. However, the lack of variation on F protein neutralising epitopes (thought to be more important targets of protective immunity) runs counter to the notion of immune driven virus evolution. The lack of difference between homologous and heterologous genotype responses suggests that G-specific neutralising responses may have been masked by cross-reactive responses to the more conserved F protein. F-specific responses have been shown to be up to 6-fold greater than G-specific neutralising responses (Olmsted *et al.*, 1986). The greater role of the F protein in development of neutralising responses is further supported by previous work that showed that only F-specific antibodies are capable of mediating complete virus neutralisation, while G-specific antibodies mediate only partial neutralisation of the virus (Anderson *et al.*, 1988, Walsh *et al.*, 1989). The lack of a genotype-specific component to the RSV neutralising response contrasts with previous work that showed that the G protein evokes genotype-specific antibody responses (Cane *et al.*, 1996, McGill *et al.*, 2004). Previous work has shown protection in the mouse model can be conferred by monoclonal antibodies with minimal *in vitro* neutralization activity (Plotnicky-Gilquin *et al.*, 1999), suggesting that failure to induce *in vitro* neutralisation, does not necessarily correlate with lack of protection. Previous studies have also shown that one such antibody (1C2) mediates *in vitro* neutralisation in the presence of complement, through Fc-dependent pathways (Mekseepralard *et al.*, 2006). However, even with the abrogation of Fc binding, this antibody provided significant protection against experimental challenge relative to controls (Mekseepralard *et al.*, 2006). These data suggest that some G-specific antibodies may be capable of inducing functional protection from natural challenge and that the

absence of neutralising ability *in vitro* does not suggest the lack of protection. A possible source of confounding in this study may be related to the use the lab-adapted strain A2. As a result of serial passaging over many years in different cell lines, it is possible that this strain has significantly deviated from wild-type strains, as has been suggested by some studies (McGill *et al.*, 2004).

The results of this study suggest that the BA genetic change that has been associated with the remarkable epidemiological success of BA variants of RSV B is not associated with loss of neutralising ability by the sera of infants infected with non-BA viruses. Although the BA genetic change is clearly associated with increased transmissibility, the data presented here show that it was not accompanied by changes on selected key neutralising epitopes on the F protein. Viewed together, these observations support the assertion that the selective advantage conferred by the BA genetic change is not associated with escape from serum neutralising responses mounted in response to infection with non-BA viruses. As mentioned above, the *in vitro* neutralisation assay used in this study is not an incontrovertible marker of protection and as such the failure to detect a differential neutralising response, does not necessarily mean that the BA genetic change did not confer an immune selection advantage. Further studies using animal models are required to clarify the role of the BA genetic change to functional protection.

It is also possible that an alternative genetic change in another part of the RSV genome occurred in tandem with the BA mutation, and may be responsible for the transmission success of the BA variants. While the transmission advantage conferred by either the BA or alternative genetic change are not associated with loss of

neutralising ability by serum antibody, it is possible that these changes may have conferred an intrinsic biological fitness advantage to BA variants relative to previous group B viruses.

Analysis of the neutralising responses of RSV A infected individuals to two RSV A test viruses A2 and Kil/A/2006 showed a similar pattern of homologous to heterologous reactivity. The infecting RSV A viruses were remarkably similar in terms of both nucleotide and amino acid identity on both the G and F proteins. Analysis of the level of variation between the G genes of RSV A test viruses by MDS showed evidence of variation between the A2 and Kil/A/2006 G genes. The Kil/A/2006 test virus was located within the cluster of RSV A infecting strains, while the A2 virus occurred on a separate region of the two dimensional MDS space. The relationship between these viruses was confirmed by phylogenetic analysis of G gene sequences. The phylogenetic tree depicting these relationships showed that while the Kil/A/2006 G gene clustered together with G gene sequences derived from infecting viruses, the A2 G gene was located on a separate branch. Viewed together, these data suggested that both the contemporary RSV A infecting viruses as well as the Kil/A/2006 test virus had evolved away from the A2 virus which had been isolated about 45 years earlier.

Despite evidence of G gene variation between the two RSV A test viruses, there was no difference in the ability of infant sera to neutralise either virus. The neutralising response to the two RSV A test viruses by sera from infants with contemporary RSV A infections both in terms of both fold rise in titre as well as the ability to seroconvert was not statistically different. These data suggest that temporal evolution of RSV does

not lead to loss of neutralising ability by serum antibody that is detectable by the neutralisation method employed. These data further suggest that RSV molecular evolution is not associated with loss of neutralisation by serum antibody. These results have implications for vaccine design. It is possible that future vaccines could act by the induction of neutralising antibody. The results presented here suggest that such vaccines are likely to retain effectiveness over a long period time without the need for recurrent antigenic updates. This might result in substantial cost benefits and potentially increase both coverage and effectiveness of future vaccine programmes in reducing the burden of illness attributable to RSV infection.

Finally, this study looked at total antibody responses to group conserved regions on the G proteins of RSV A and B. The results presented show that antibody responses directed at these regions are strongly group-specific. The data presented support the results of previous studies that showed that immunisation of mice with similar peptides resulted in group-specific protection (Simard *et al.*, 1997). These results also provide further support to the idea that the RSV neutralising response is strongly group-specific, suggesting the need to consider the parallel development of live attenuated vaccines based on an RSV B backbone in order to ensure that protective immunity that is induced through vaccination is effective against genotypes from both groups of RSV.

Chapter 8 - Cytokine/chemokine responses following natural infection in infants

Introduction

RSV is the most important viral cause of severe pneumonia in infancy (Nair *et al.*, 2010). Despite this, the mechanisms of pathogenesis among infants who are naturally infected are not fully understood. The first point of contact between RSV and the host is the epithelium of the upper respiratory tract. Since RSV replication occurs mainly in airway columnar epithelial cells, cytokine/chemokine responses generated at this site are likely to significantly influence the balance between virus clearance and pathology.

RSV infection of epithelial cells leads to significant up-regulation of innate immune receptors such as Toll Like Receptor 4 (TLR-4) within 24 hours of infection (Xie *et al.*, 2009). TLR-4 and CD14 on human monocytes have been shown to bind to the F protein leading to the production of pro-inflammatory cytokines TNF-α, IL-6 and IL-12 (Kurt-Jones *et al.*, 2000). In human infants, TLR4 is up-regulated at the acute phase of RSV infection and down-regulated at the convalescent stage, underpinning its central role in the recognition of RSV and in the initiation of host antiviral responses (Gagro *et al.*, 2004). RSV infection also promotes the secretion if TNF-α, IL-6, MCP1 and RANTES through interaction with TLR-2 and TLR 6 expressed on leukocytes (Murawski *et al.*, 2009). Human alveolar macrophages have also been shown to be a potent source of pro-inflammatory cytokines/chemokines TNF-α, IL6 and IL8 (Becker and Soukup, 1999).

Peripheral production of pro-inflammatory cytokines/chemokines has been linked to initiation of clinical symptoms such as fever and malaise through activation of neurological pathways (Watkins *et al.*, 1995), providing grounds to suggest that rises in pro-inflammatory cytokine/chemokine responses may be related to illness following natural RSV infection.

Once within human epithelial cells, RSV induces further production of IL-8 (Harrison *et al.*, 1999, Arnold *et al.*, 1994) as well as the C-C chemokines RANTES, MCP1 and MIP-1α in a dose dependant manner (Olszewska-Pazdrak *et al.*, 1998). RANTES has been shown to induce the migration of CD4+ T cells and monocytes (Schall *et al.*, 1990) to the site of infection. MIP1-α is thought to induce the chemotaxis of B cells, CD8+ T cells and CD4+ T cells (Schall *et al.*, 1993) and has further been suggested to have a role in eosinophil degranulation (Harrison *et al.*, 1999). MCP1 on the other hand induces the migration of both CD8+ and CD4+ T cells (Loetscher *et al.*, 1994).

The factors that promote the development of severe disease in infants have not been clearly defined. Among RSV-infected infants admitted to hospital with severe illness, the levels of RANTES, MIP-1α, IL-6, IL-8 and IL-10 are significantly higher compared to non-RSV controls (Sheeran *et al.*, 1999), suggesting that these cytokines/chemokines have a role to play in RSV pathogenesis in infancy. There is evidence in the murine model that shows that RSV infection induces simultaneous production of both inflammatory (IFN-γ) and regulatory (IL-10) cytokines in the acute phase of infection (Sun *et al.*, 2011). It has been suggested that the simultaneous production of these antagonistic cytokines is important in achieving a

balance between virus clearance and prevention of immune mediated pathogenesis (Sun *et al.*, 2011, Loebbermann *et al.*, 2012).

The role of virus-specific factors on the early host response to RSV has not been exhaustively investigated. Murine studies have shown that some strains of the virus tend to induce more severe pathology than others. For example comparison of the pathogenesis induced by infection of BALB/c mice with either the A2 or line 19 strains, showed that line 19 induced airway hyper-reactivity and mucus overproduction while A2 did not (Lukacs *et al.*, 2006). In this model, disease severity correlated with levels of IL-13 since infection of IL13-/- mice with line 19, abrogated airway hyperreactivity (Lukacs *et al.*, 2006). Later studies showed that certain mutations in the F protein are strongly associated with the ability of line 19 to induce airway hyperresponsiveness and enhanced mucus production (Moore *et al.*, 2009), raising the possibility that natural variation within this protein in wild-type isolates may similarly play a role in differential disease outcome in infants.

In this chapter, cytokine/chemokine responses following natural RSV infection in infants were explored. Cytokine/chemokine responses over the entire course of mild RSV infection as well as among infants admitted with severe RSV related pneumonia were related to disease outcome, viral load and different strains of RSV.

Chapter Aims

This chapter will explore the relationship between mild RSV infection and different cytokine/chemokine concentrations with a view to identifying cytokines/chemokines that are associated with development of disease following natural infection. The effect

of viral load on the expression levels of different cytokines/chemokines will also be investigated. To evaluate whether other respiratory viruses induce cytokine/chemokine responses that vary from those induced by RSV infection, the levels of different cytokines/chemokines during RSV and rhinovirus infection will be compared. This chapter will also explore the kinetics of different cytokine/chemokine responses following natural infection in the household. Cytokine/chemokine responses in the nasal secretions of seven infants from whom nasal samples were collected at least twice weekly over the course of infection will be investigated. The relationship between peaks in cytokine/chemokine response, presence or absence of Upper Respiratory Tract Infection (URTI) symptoms (i.e. cough, sore throat, runny nose and nasal congestion) and viral load will be explored. The effect of virus specific factors on the infant cytokine/chemokine response will be investigated by comparing cytokine/chemokine responses in the nasal secretions of infants infected with either RSV A or B. Finally, the effect of different cytokine/chemokine concentrations on different clinical phenotypes of severe pneumonia will be investigated.

Methods

The samples used in this study were derived from two studies of natural RSV infection in infancy and early childhood. The first set of samples were derived from a longitudinal household study in which nasal flocked swab samples were prospectively obtained from infants during an RSV epidemic at twice weekly intervals. Diagnosis of RSV (both A and B) along with 14 other respiratory pathogens was carried out on each sample using a multiplex real time PCR. In the studies whose results are reported in this chapter, only samples obtained a few days prior to, during and shortly after cessation of RSV shedding are included for analysis, and not the entire sample set

collected over the six month period during which active surveillance was undertaken. Further details on the household study design can be found in chapter 4.

The second set of samples was derived from paediatric hospital inpatients admitted with WHO syndromically defined severe or very severe pneumonia and a laboratory confirmed RSV diagnosis. Nasal wash and nasopharyngeal aspirate samples were collected from these infants and were used in the detection of different cytokines/chemokines in this study. Further details on the design of the clinical surveillance study from which these samples were obtained can be found in chapter 4.

A total of 100 samples collected from seven infants from the household study are included in the analysis described in this chapter. Each of the infants in this study was less than 1 year of age at the time of entry into the study. Furthermore, 86 samples from infants and young children admitted to KDH with RSV-associated severe or very severe pneumonia were included in the study. Viral load was approximated by calculating the inverse of cycle threshold (ct) values obtained from real-time PCR diagnostic assays. The laboratory assays used in the measurement of cytokine/chemokine concentrations are described in detail in chapter 4.

Comparison of cytokine/chemokine concentrations between different groups of was done using a two sided student t test based on $\log_{10}$ normalised concentrations. Samples whose cytokine/chemokine concentrations were below the lower limit of detection, were assigned the value 0.0001 pg/ml to enable log transformation and statistical analyses. In almost all samples tested, the levels of IL-5, IL-13, IL-4, IL-2 and IL-12p70 were below the lower limit of detection and as a result, these

cytokines/chemokines were not subjected to statistical analyses. To describe the kinetics of different cytokine/chemokine responses, differences between mean cytokine/chemokine concentrations at different time points pre/post infection were measured using multiple regression. In these analyses, cytokine/chemokine concentrations were the dependent variable while the different time points pre/post infection were the independent variable.

Results

Cytokine/chemokine Responses in the Longitudinal Household study

Diversity of respiratory pathogens detected

In total, cytokine/chemokine levels were evaluated in 100 samples derived from different infants in the household study. Figure 8.1 shows the distribution of different respiratory pathogens in the study samples that were evaluated. Of the 16 pathogens that were included in the detection panel, only 8 were found to be present in the samples included in this study. The pathogens that were present at the highest proportions were RSV A (14%), rhinovirus (21%) and RSV B (22%).

Discussion

In this chapter, the neutralising antibody responses in the acute and convalescent phase sera of children with a positive RSV diagnosis and who were admitted with severe pneumonia are presented. The infants and young children in this study were recruited over the course of 3 RSV epidemics in Kilifi. The group designations of each infecting strain was characterised using previously described molecular techniques (Scott *et al.*, 2004) in addition to F and G gene sequencing of a number of infecting viruses. In addition to molecular sequencing and genotyping of infecting strains, comprehensive clinical phenotyping of clinical pneumonia was also carried out for each of the paediatric pneumonia admission cases recruited in this study.

The availability of comprehensive clinical and molecular diagnostic data in this study is an improvement over previous studies that have sought to look at the infant neutralising response. Studies done in the 1960s failed to show a difference between homologous and heterologous antibody responses in the sera of acutely infected infants (Coates *et al.*, 1963, Wulff *et al.*, 1964). These studies were done on the presumption that there was homotypic strain exposure in any single epidemic, a premise that has since been rendered invalid by studies that show evidence of co-dominance of RSV A and B strains in some epidemics (Hendry *et al.*, 1989, Freymuth *et al.*, 1991). Subsequent studies that looked at the group-specificity of the neutralising response failed to show a statistically significant difference between the homologous and heterologous response to both RSV A and B (Muelenaer *et al.*, 1991, Hendry *et al.*, 1988).

In order to assess the magnitude of the serum neutralising response following infection, mean acute and convalescent antibody titres to RSV test strains were compared. An overlay of the acute and convalescent phase neutralising antibody titre distributions (shown in figure 5.4) showed that in general, the acute antibody titre was lower than the convalescent antibody titre, suggesting that infants and children generally mount a strong neutralising antibody response following natural infection. When the acute and convalescent phase distributions in three age classes (0-5, 6-11 and 12 months of age and above) were compared there was evidence that age plays an important role in the development of the neutralising antibody response. Whilst there was a modest difference between the acute and convalescent phase titres in the 0-5 month age class, in the older age classes (6-11 and 12+ months of age) there was evidence of a robust neutralising response at convalescent phase of infection relative to the acute phase (figure 5.5). These data suggest that in general infants mount neutralising responses to natural infection and this response tends to increase with age.

It has been suggested that the inability of very young infants to mount a strong neutralising response to infection is related to high titres of maternally-derived antibody present in very young infants (Murphy *et al.*, 1986b). To explore the relationship between pre-existing antibody and the ability to mount a robust response to infection, correlation analysis was carried out between the magnitude of the neutralising response and the titre of pre-existing antibody. The results of Spearman's rank correlation analysis showed that there was a strong and statistically significant negative association between the magnitude of the neutralising response (measured as fold rise in titre) and the titre of acute phase antibodies. A similar pattern was seen

when acute phase titres were stratified into four quartiles and the proportion of infants seroconverting in each quartile determined. Over 90% of the infants in the first quartile seroconverted, relative to 59.6%, 13.5% and 0% who seroconverted in the 2nd, 3rd and 4th quartiles respectively. These data strongly suggest that high titres of pre-existing antibody interfere with the infants' native response to infection. These data accord with a previous report by Parrot *et al.* who showed that the convalescent phase neutralising response to infection was negatively associated with the acute phase titre (Parrott *et al.*, 1973).

In addition to the interfering effect of pre-existing antibody on the natural infant response to infection, it has also been suggested that young age may independently account for the relatively poor neutralising antibody response mounted by very young infants. A study by Murphy *et al.* found that age primarily affects the response to the F protein while pre-existing antibodies affect the response to the G protein (Murphy *et al.*, 1986a). Since neutralising responses are mainly targeted at the F protein this age related reduction in response to the F protein could possibly be reflected in the overall neutralising response. The data presented in this study shows that there was a moderate but statistically significant positive association between age and the magnitude of the neutralising response. However, analysis of the proportion of infants who seroconverted within different age classes (Figure 5.8) showed that a small proportion of even the youngest infants mounted a seroconverting response to infection, suggesting that even very young infants are capable of mounting a strong neutralising response to natural infection. This suggests that the key driver of the poor infant neutralising response is likely to be the presence of pre-existing maternal antibodies and not age. This idea is supported by the work of Shinoff *et al.* who found

that pre-existing antibody and not age is the key determinant of the infant neutralising response (Shinoff *et al.*, 2008).

A number of studies have looked at the protective effect of neutralising antibodies against severe disease in young infants. Although some studies have shown evidence of protection from severe disease by antibodies (Glezen *et al.*, 1981a), some studies have reported that maternally-derived neutralising antibodies are not associated with protection from severe disease (Bulkow *et al.*, 2002). The data presented in this chapter show a protective association between neutralising antibodies and some clinical features of severe pneumonia. Out of seven clinical feature that were analysed, neutralising antibodies were found to be associated with a lower risk of developing shock, flaring, hypoxia and crackles, although statistical significance was only reached in the case of shock. These data generally suggest that neutralising antibodies, even in this group of severely ill children are associated with protection from some features of clinical pneumonia.

A number of studies have looked at the sequence characteristics of different strains of RSV both at the F and G gene levels. Comparison of the G protein genes of two RSV A strains, A2 and Long found 94% amino acid sequence identity between these two strains (Johnson, 1987). However the A2 G protein gene only shared 53% amino acid identity with the G protein of the group B strain, 18537 (Johnson, 1987). Sullender *et al.* found that another group B strain, 8/60 shared 98% amino acid identity with the 18537 strain, but only shared 56% amino acid identity with the A2 strain (Sullender *et al.*, 1990). These results strongly suggested that in general, the G protein is highly variable between the two groups but is relatively conserved within each group. In

contrast, comparison of the deduced amino acid sequences of the 18537 and A2 F proteins showed that they shared 91% amino acid identity (Johnson and Collins, 1988), suggesting that the F protein is highly conserved between groups. The data presented in this chapter shows that there was 87% amino acid identity in the deduced F protein amino acid sequences of infecting RSV A and B isolates and only 28.6% amino acid identity at the G protein level. These data are therefore in general agreement with previous findings and reinforce the idea that the F protein is highly conserved between the 2 groups while the G protein is highly variable. Despite the high level of sequence conservation of the F proteins of wild-type isolates, neutralising responses to this protein do not appear to correlate with protection from natural (Glezen *et al.*, 1986) or experimental (Hall *et al.*, 1991) re-infection. It is possible that despite its relatively greater level of conservation, neutralising antibody epitopes on the F protein may be located on a small but variable part of the protein.

To explore this possibility, four neutralising antibody epitopes on the F protein were identified in the literature (West *et al.*, 1994, Martin-Gallardo *et al.*, 1991, Lopez *et al.*, 1990, Trudel *et al.*, 1987a) and mapped on the deduced amino acid sequences of infecting RSV A and B strains. The data presented in figure 5.12 show that two of these epitopes; aa205 -225 (Bourgeois *et al.*, 1991) and aa221- 236 (Trudel *et al.*, 1987a) were located on a region of the F protein that contained a number of group-specific amino acids. In other words some amino acid residues within these epitopes were perfectly conserved within but not between the two groups. There are studies that have shown that such differences could substantially influence the group-specificity of the neutralising antibody response. West *et al.* for example, demonstrated that one monoclonal antibody, RS348 that was generated against the A2

strain (RSV A) (West *et al.*, 1994) and whose target is aa205 -225 on the F protein (Bourgeois *et al.*, 1991) mediated a 782-fold greater neutralisation of homologous virus (RSV A) relative to heterologous virus (RSV B) (West *et al.*, 1994). Analysis of the amino acids of this epitope among the infecting strains presented in this study (figure 5.12) showed that it contained two amino acids that were conserved within but not between the two RSV groups. This strongly suggests that if the infant response similarly targets this neutralising epitope, it is likely that such a neutralising response would be highly group-specific.

A similar pattern of group-specific neutralisation has been observed using a neutralising monoclonal antibody, 7C2 (West *et al.*, 1994) whose target is aa221-236 (Trudel *et al.*, 1987a) on the F protein. Analysis of the patient isolates in this study showed that aa221-236 contained one amino acid that was conserved within but not between the two RSV groups. The 7C2 monoclonal antibody targeting this region has been shown to mediate a 2.5 fold greater homologous vs. heterologous neutralisation at group level (West *et al.*, 1994), supporting the idea that infant responses targeted at this region would be similarly group-specific.

The final two neutralising epitopes that were mapped on the deduced F protein amino acid sequence alignments of infecting strains were perfectly conserved in all infecting strains irrespective of whether they belonged to group A or B. Previous studies using a neutralising monoclonal antibody targeting one of these epitopes (aa262-268) have shown that it mediates a 1.4-fold greater homologous to heterologous neutralisation (West *et al.*, 1994), suggesting that neutralising responses directed at this region are largely cross-reactive.

In summary, these data suggest that neutralising responses to the F protein are a mixture of both group-specific and group cross-reactive responses and that amino acids that are conserved within but not between groups could potentially lead to an overall greater homologous to heterologous neutralising response.

This study finally looked at the group-specificity of the neutralising antibody response. Responses to contemporary strains of RSV A and B were used using acute and convalescent sera obtained from infants whose infecting strains were well characterised by molecular techniques. The results of these analyses show that the proportion of homologous seroconversion was greater than that of heterologous seroconversion irrespective of whether the infecting strain was RSV A or B. The effect of age on the ability to mount a strong homologous vs. heterologous response was also investigated. Infant responses were stratified into 3 age classes, 0-5, 6-11 and 12+ months of age and their ability to neutralise homologous virus was compared to their ability to neutralise heterologous virus. The results of these analyses showed that in all three age classes the mean response to homologous virus (in terms of fold rise in titre) was significantly greater than the mean response to heterologous virus.

The weakest homologous responses to RSV A and B occurred in 0-5 month age class. It is possible that the inability to mount a strong neutralising response within this age class may be related to the presence of pre-existing maternal antibody as discussed earlier. On the other hand, the strongest neutralising responses to either homologous or heterologous virus were seen in the 6-11 month age class. This robust response might be attributed to the absence of pre-existing maternally-derived antibody at the

time if infection. Analysis of the dynamics of the acute phase neutralising response in this study showed that maternally-derived antibodies had declined to minimal levels by 3 months of age suggesting that infants exposed to primary infection in the 6-11 month age class would have largely been free of maternal antibody. Analysis of the heterologous responses in this age class showed that although the mean heterologous responses (in terms of log fold rise in titre) to both RSV A and B were high relative to the 0-5 month age class, they were nonetheless below the 4-fold threshold that has traditionally been used as a measure of a significant serological response.

It was further observed that although the homologous responses to RSV A and B in the 12+ month age class were above the 4-fold threshold, they were nonetheless lower than the homologous responses in the 6-11 month age class. A possible explanation for this could be that at this age (12 months and above), young children in their second year of life are likely to be undergoing secondary infection. The inability to detect a homologous response in the 12+ month age class that was of comparable magnitude to that in the 6-11 month age class may be attributed to the presence of relatively high levels of pre-existing antibody brought about by either (i) maintenance of high antibody levels following primary infection or (ii) an extremely rapid rise of secondary antibody shortly after exposure. This would imply that the acute sample – which is collected after the onset of symptoms and therefore a few days after actual infection – would be partly reflective of the antibody response to the current infection. The dynamics of the primary and secondary neutralising antibody responses are considered in greater detail in chapter 6.

In summary, the data presented in this chapter explore the neutralising response to RSV and more specifically, the group-specificity of the neutralising response in a group of severely infected infants. The data show that the neutralising antibody response is strongly group-specific and that group-specific amino acid differences in the neutralising epitopes on both the F and G proteins of the infecting strains may be associated with this specificity. The data presented in this chapter supports the assertion that population level group-specific immunity may underlie the observed group replacement dynamics of RSV.

Chapter 6 - Kinetics of the neutralising antibody response

Introduction

RSV re-infects throughout life (Henderson *et al.*, 1979, Glezen *et al.*, 1986). The ability to repeatedly infect may be related to antigenic variation or short-lived protective immunity or both. Studies of the antigenic characteristics of RSV strains that cause repeat infections suggest that antigenic variation does not play a central role in the ability of RSV to re-infect (Beem, 1967) although there is evidence that shows that re-infection with genetically variant strains is a common phenomenon (Agoti *et al.*, 2012). The duration of protective immunity post natural infection has not been clearly defined, although human experimental challenge studies show that re-infection can be induced within 2 months of the primary challenge (Hall *et al.*, 1991). The correlates of protective immunity in terms of both the duration and strain-specificity of the immune responses induced by natural infection are an important subject of research since they will provide insights on the likely effectiveness of future RSV vaccines.

The mechanisms of antibody-mediated protection have been studied for a number of other acute viral infections. Infection with viruses such as the measles virus, poliovirus and the mumps virus induce extremely long lived antibody responses whose respective half lives are estimated to be over 200 years (Amanna *et al.*, 2007). Infections such as poliovirus induce strain-specific antibodies that are maintained for a number of years in the absence of natural re-exposure (Paul and Riordan, 1950, Paul *et al.*, 1951). On the other hand, natural influenza A infection has been shown to

induce neutralising antibodies that decline to pre-infection levels within one year of infection (Buchy *et al.*, 2010). Studies of the duration of the total antibody response to natural primary RSV infection suggest that RSV-specific antibodies are short-lived. Welliver *et al.* showed that the serum antibody response to RSV declined to pre-infection levels by one year after primary infection (Welliver *et al.*, 1980), while the secretory IgA response declined to pre-infection levels by 3 months post infection (Kaul *et al.*, 1981). There are currently no data available that show the duration of neutralising antibody responses in the serum of infants with natural primary RSV infection.

The determinants of the variation in the duration of antibody induced protection between different viral infections are not fully understood. A number of murine based studies have shown that antibody producing plasma cells can be maintained for long periods of time in the absence of antigenic re-stimulation (Slifka and Ahmed, 1998), suggesting that that these cells may be a key source of long lived antibody responses. However not all plasma cells are long lived (Slifka and Ahmed, 1996). There is some evidence that suggests that the longevity phenotype oF-specific plasma cell populations is associated with their cognate epitopes (Slifka and Ahmed, 1998), meaning that the long term maintenance of antibody responses through this mechanism is epitope - and by extension - virus specific. These observations agree with the ability of some viral infections to induce short-lived antibody responses while others such as those mentioned above appear to induce responses that are maintained for many years.

Despite the evidence of short-lived primary antibody responses, it is well recognised that severity of RSV disease declines significantly upon secondary infection (Glezen *et al.*, 1986, Nokes *et al.*, 2008). While it is likely that the maturation of the respiratory system with age plays a crucial role in moderating disease severity, a number of studies have provided indirect evidence that maturation of the respiratory system alone is not sufficient to protect from severe disease. RSV is known to be a major cause of severe respiratory illness among the elderly and adult organ transplant recipients who are under immunosuppressive therapy to reduce the likelihood of organ rejection (Falsey *et al.*, 2005, Krinzman *et al.*, 1998). Since severe disease frequently occurs in these adult populations, physiological maturity of the airways alone cannot account for protection from severe disease. These data therefore suggest that protection from severe disease in healthy, immuno-competent individuals may be associated with the ability to generate a rapid, protective neutralising response shortly after re-infection.

The role of maternal antibodies in protecting from severe infection has been extensively studied. Although there are some contradicting data (Bulkow *et al.*, 2002), it is generally agreed that maternal neutralising antibodies provide protection from severe disease in early infancy (Glezen *et al.*, 1981b). It has previously been shown that the total maternal antibody response is relatively short-lived with a half life of less than three months (Ochola *et al.*, 2009). It has also been suggested that the duration of protection by maternal antibodies is related to the titre at birth, and that higher cord titres are associated with a longer duration of protection from natural infection while lower cord titres protect for a shorter duration (Ogilvie *et al.*, 1981).

There is a paucity of data however on the mean duration of protective neutralising antibodies above thresholds that are commonly associated with protection from severe disease.

Chapter Aims.

This chapter explores the serum neutralising antibody responses in a birth cohort that was followed for approximately 30 months or until three RSV epidemics had been experienced. The cohort was recruited in two phases, which are referred to in this chapter as cohort 1 and cohort 2. The chapter aims to explore the relationship between exposure to infection, inferred through incidence data obtained from inpatient diagnostic records and temporal variations in the mean neutralising antibody titres in the two cohorts. The chapter also aims to define the duration of neutralising antibody response following primary infection. The mean neutralising responses at different time points post infection will be compared to that of a pre-exposure control group. The chapter further aims to define the mean duration of protective neutralising antibodies of maternal origin using a putative protective threshold obtained from the literature. Using this threshold, the mean duration of protective maternal antibodies as well as the age prevalence of protective maternal neutralising antibodies will be calculated. Finally the dynamics of the primary and secondary neutralising responses will be evaluated, by comparing neutralising titres in sera collected within 10 days of identification of primary and secondary infection to a pre exposure control group.

Materials and methods

Study population and design

The study used archived serum and nasal wash samples collected from a birth cohort of children recruited between 2002 and 2003 in the rural District of Kilifi on the Kenyan Coast. The birth cohort was recruited in 2 phases: phase 1 was recruited between January and May 2002 while phase 2 was recruited between December 2002 and July 2003. At delivery, a cord blood sample was taken, followed by routine blood samples scheduled at 3-monthly intervals until each child had experienced three RSV epidemics or was lost to follow-up. Through home or clinic surveillance nasal washes were collected from children that displayed symptoms of acute respiratory infection and detection of RSV done using IFAT. For positive samples the RSV group was determined by Reverse Transcriptase (RT)-PCR (Stockton, 1998). An acute blood sample was collected as soon as possible after RSV diagnosis and a convalescent blood sample was collected about one month later. 28 individuals from the birth cohort from whom at least eight serum samples had been collected over the course of follow-up were included this study. All had a virus confirmed primary infection during the course of the study, while 9 had virus confirmed secondary infections. Of the 28 primary infections, 16 were group A infections while 12 were group B. Of the 9 virus confirmed secondary infections, 3 were RSV A infections while 6 were RSV B. Out of the 3 individuals who had RSV A secondary infections, 1 had a primary RSV A infection while 2 had RSV B primary infections. Similarly, out of the 6 individuals who had RSV B re-infections, 3 had primary RSV A infections while 3 had primary RSV B infections. In all the studies outlined in this chapter, serum neutralising antibodies were measured to local RSV A and B strains (Ken/A/2006

and Ken/B/208) and a mean titre calculated. Antibodies were measured in acute and convalescent sera as well as in the cord and routine 3 monthly sera. A pre exposure control group consisting of sera collected up to six months before infection from children who were older than 5 months of age at the time of collection (pre-exposure control group) was defined for comparison. Exposure in the presence study was estimated using paediatric inpatient RSV surveillance data. The study population, design and diagnostic techniques have been described elsewhere (Nokes *et al.*, 2009).

RSV epidemic start and end dates during the period of surveillance were adopted from the work of Okiro (E. Okiro PhD Thesis) and are shown in table 6.1.

Year	*epidemic*	*start week*	*finish week*
2002	1	11	**26**
	2	49	**52**
2003	2	1	**15**
2004	**3**	**2**	**22**

Table 6.1 Epidemic start and end weeks over the period of follow up of the 2 birth cohorts. These definitions are adopted from the work of Okiro (Emelda Okiro thesis, 2007).

Statistical analyses

Data were analysed using Stata (version 11, Statacorp, College station Texas). For the purpose of calculating the duration of the neutralising response, the start of the host immune response to RSV was assumed to coincide with the date of collection of nasal samples that turned out to be positive for RSV antigen by IFAT. It was further assumed that antibody responses had declined to pre-infection levels if they were

similar to or fell below the mean pre-exposure control titre. The duration of the neutralising antibody response following primary infection was determined using a multiple linear regression model. In this model the neutralising antibody titres were the dependant variable while the number of months before or after infection and age were the independent variables. Acute and convalescent phase responses during primary and secondary infection were also compared using a multiple linear regression model. The rate of decay of maternal neutralising antibody was analysed by comparing infant titres to a neutralising antibody titre of 2.48 $\log_{10}ND_{50}$ that has previously been associated with protection against severe disease (Glezen *et al.*, 1981a). Time to failure survival analysis was used to determine the average duration of protective maternal antibodies. Failure was defined as a decline of maternal titres below the titre associated with clinical protection. The average duration of neutralising antibody protection by maternal antibodies was obtained by calculating the reciprocal of the average rate of decline of maternal titres obtained from survival analysis. The analytical output for the data presented in this chapter is presented in appendix 2.

Results

General age dynamics of the neutralising response

To describe the age dynamics of the neutralising response in the first two years of life, serum neutralising antibodies from the 2 birth cohorts were combined and the mean neutralising response calculated in different age classes. Ten age classes were defined for these analyses; 0-2 (n=49), 3-5 (n=31), 6-8 (n=22), 9-11 (n=32), 12-14 (n=36), 15-17 (n=29), 18-20 (n=21), 21-23 (n=22), 24-26 (n=28) and 27+ (n=10) months of age. There was a sustained decline of neutralising antibodies in the first 8 months

of life, followed by an increase in mean neutralising titres between the ages of 11 and 20 months. There was a sustained increase in titre between the ages of 21 to 26 months. This was followed by a slight decline among children who were above 27 months of age. These data are graphically represented in figure 6.1.

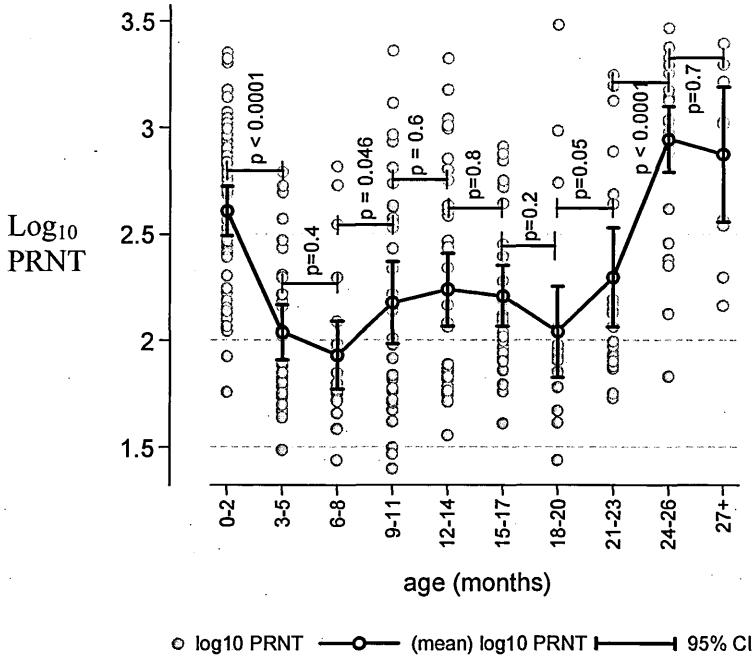

Figure 6.1 The age dynamics of the neutralising antibody titre in the RSV birth cohort. Infant neutralising antibody titres are stratified into 10 age classes (x-axis) and mean neutralising titres (y-axis) compared in successive age classes. The grey circles indicate the distribution of actual titres within respective age classes while the lines cutting across the mean values (open cirles) indicate 95% confidence intervals. The p values cutting across different age classes indicate whether the differences between the means of the groups being compared are statistically significant.

The relationship between exposure and the neutralising antibody response

RSV inpatient surveillance data were used to estimate the periods when RSV epidemics were occurring within the community. Infants were considered to be exposed to and at risk of infection during epidemic periods and at lower or no risk of infection during inter-epidemic periods. To explore the relationship between the

neutralising response and exposure, neutralising antibody titres from 19 infants in cohort 1 and 9 infants in cohort 2 whose sera were collected serially over approximately 30 months of follow up were plotted against RSV incidence data obtained from inpatient surveillance. The data in figure 6.2 shows the result of this comparison.

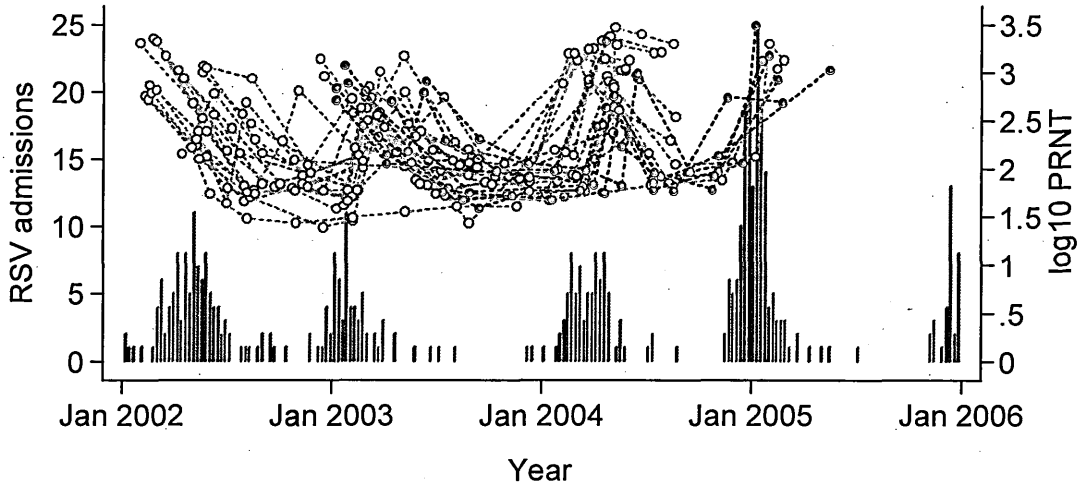

Figure 6.2 The dynamics of the neutralising antibody response relative to population level transmission of RSV. The blue bars show the number of paediatric (<5 yrs old) RSV cases in severe and very severe pneumonia admissions to Kilifi District Hospital over the follow up period. The dashed grey and dashed red lines indicate temporal dynamics of the neutralising response in cohort 1 and 2 respectively. 19 individuals from cohort 1 and 9 individuals from cohort 2 were included in this analysis.

To further explore the relationship between population level virus transmission and variation in the mean neutralising response, serum samples from cohort 1 and 2 were stratified by date of collection into 11 and 10 strata respectively of 3 calendar months each. 19 and 9 individuals from cohorts 1 and 2 respectively were included in these analyses. The mean neutralising titre within each stratum was then calculated and

compared to that of the subsequent stratum. Figure 6.3 is a summary of the mean neutralising response within each stratum in cohort 1 relative to population level transmission. Table 6.2 contains the mean age, dates of sample collection as well as total number of samples in each stratum. As an estimate of the level of exposure, the proportion of samples that were collected within and outside epidemics are provided for each stratum. The mean neutralising antibody titre in stratum 1 (2.8 $\log_{10}$ PRNT) was significantly greater than the mean neutralising titre in stratum 2 (2.27 $\log_{10}$ PRNT, $p<0.0001$). 36% and 71% of the samples in strata 1 and 2 respectively were collected during epidemic 1 while the remaining samples were collected during inter-epidemic periods. The mean neutralising response in stratum 2 was on the other hand significantly greater than the mean response in stratum 3 (1.92 $\log_{10}$ PRNT, $p=0.012$), where all the samples were collected in the inter-epidemic period between epidemics 1 and 2. There was no significant difference between the mean titre in stratum 3 and 4 (1.92 $\log_{10}$ PRNT vs. 1.86 $\log_{10}$ PRNT; $p=0.74$). There was a statistically significant difference between the mean neutralising titre in strata 4 and 5 (1.92 $\log_{10}$ PRNT vs. 2.34 $\log_{10}$ PRNT; $p=0.002$). 31% and 96% of the samples in strata 4 and 5 respectively were collected in the course of epidemic 2. There was no statistically significant difference between the mean neutralising responses in strata 5 and 6 (2.34 $\log_{10}$ PRNT vs. 2.33 $\log_{10}$ PRNT; $p=0.9$). All the samples in stratum 6 were collected in the inter-epidemic period between epidemics 2 and 3. The mean neutralising response in stratum 7 was borderline significantly lower than the mean neutralising response in stratum 6 (2.33 $\log_{10}$ PRNT vs. 2.03 $\log_{10}$ PRNT; $p=0.06$). All the samples collected in stratum 7 were collected in the inter-epidemic period between epidemics 2 and 3. The mean neutralising response in stratum 8, where 22% of the samples were collected in the course of epidemic 3, was not significantly

different from the mean neutralising response in stratum 7 (1.97 $\log_{10}$ PRNT vs. 2.03 $\log_{10}$ PRNT; p=0.73). However the mean neutralising titre in stratum 9 where all the samples were collected during epidemic 3 was significantly greater than that of stratum 8 (2.66 $\log_{10}$ PRNT vs. 2.03 $\log_{10}$ PRNT; p<0.0001). There was no statistically significant difference between the neutralising responses of strata 9 and 10 (2.66 $\log_{10}$ PRNT vs. 2.92 $\log_{10}$ PRNT; p=0.07) and 10 and 11 (2.92 $\log_{10}$ PRNT vs. 2.78 $\log_{10}$ PRNT; p=0.5). These data are summarised in figure 6.3.

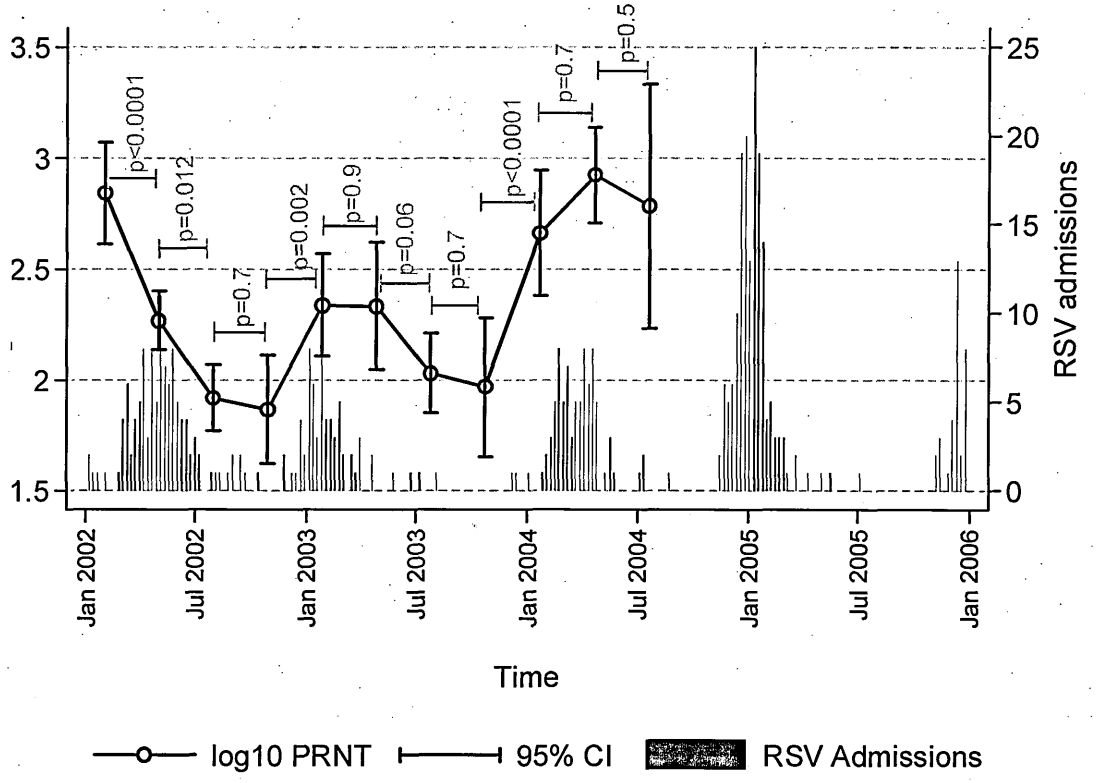

Figure 6.3 The dynamics of the neutralising response in cohort 1 relative to incidence of RSV. Sera from 19 individuals who were prospectively recruited and followed up for approximately 30 months are included in this analysis. Eleven strata consisting of 3 calendar months each were generated and the mean neutralising titre in each stratum calculated. The mean titre in each stratum was compared to that of the subsequent stratum. The p values shown indicate whether there was a significant difference between the mean titres of the different strata being compared. The titres used in this analysis were mean titres obtained using two local test viruses (Ken/A/2006 and Ken/B/2008). The numbers within each stratum are shown in table 6.2

Stratum	First date	Last Date	Mean age - Months (range)	n	% within epidemic
1	01-Feb-02	18-Apr-02	0.2 (0-1.3)	14	36
2	02-May-02	30-Jul-02	3.3 (2.3-5.7)	24	71
3	02-Aug-02	28-Oct-02	6 (5.5-8.3)	17	0
4	30-Oct-02	22-Jan-03	8.6 (8.3-10.2)	13	31
5	29-Jan-03	23-Apr-03	11.8 (10-14.1)	23	96
6	06-May-03	23-Jul-03	15 (14.3-17.3)	14	0
7	31-Jul-03	14-Oct-03	17.9 (17.1-20.3)	15	0
8	29-Oct-03	15-Jan-04	20.8 (20.1-23.2)	9	22
9	23-Jan-04	19-Apr-04	24 (23.1-26.3)	18	100
10	23-Apr-04	14-Jul-04	26.7 (25.3-29.3)	19	74
11	27-Jul-04	20-Aug-04	29.4 (29.1-29.9)	5	0

Table 6.2 The beginning and end dates of each stratum shown in figure 6.3 as well as the mean age and total number of samples for each stratum are shown in this table. As a measure of the level of exposure in each stratum, the proportion of samples that were collected during an epidemic are also indicated

Figure 6.4 contains a summary of the mean neutralising response relative to transmission within cohort 2, while table 6.3 contains the mean age, dates of sample collection as well as total number of samples in each stratum. The mean neutralising response in stratum 1 was significantly greater than that in stratum 2 (2.95 $\log_{10}$ PRNT vs. 2.45 $\log_{10}$ PRNT; p=0.02). All the samples in strata 1 and 2 were collected during epidemic 2. There was no significant difference between the mean neutralising responses in strata 2 and 3 (2.45 $\log_{10}$ PRNT vs. 2.25 $\log_{10}$ PRNT; p=0.3). However there was a significant difference between the mean titres in strata 3 and 4 (2.25 $\log_{10}$ PRNT vs. 1.84 $\log_{10}$ PRNT; p=0.026). All samples in strata 3 and 4 were collected in

the inter-epidemic period between epidemics 2 and 3. There was no statistically significant difference between the mean neutralising titres in strata 4 and 5 (1.84 $\log_{10}$ PRNT vs. 1.81 $\log_{10}$ PRNT; p=0.88), nor was there a difference between the mean titres of strata 5 and 6 (1.81 $\log_{10}$ PRNT vs. 1.93 $\log_{10}$ PRNT; p=0.46). 17% and 100% of the samples in strata 5 and 6 were collected in the course of epidemic 3. There was however a statistically significant difference between the mean neutralising titres in strata 6 and 7 (1.93 $\log_{10}$ PRNT vs. 2.53 $\log_{10}$ PRNT; p<0.0001). 67% of the samples in stratum 7 were obtained during epidemic 3. There was similarly a significant difference between the mean responses of strata 7 and 8 (2.53 $\log_{10}$ PRNT vs. 1.91 $\log_{10}$ PRNT; p=0.001). All samples in stratum 8 were collected in the inter-epidemic period between epidemics 3 and 4. There was no significant difference between the mean responses in strata 8 and 9 (1.91 $\log_{10}$ PRNT vs. 2.22 $\log_{10}$ PRNT; p=0.12), but there was a significant difference between the mean responses in strata 9 and 10 (2.22 $\log_{10}$ PRNT vs. 3.05 $\log_{10}$ PRNT; p<0.0001). 30% of the samples in stratum 9 were collected in the course of epidemic 4 while 71% of the samples in stratum 10 were collected in epidemic 4. These data are graphically presented in figure 6.4.

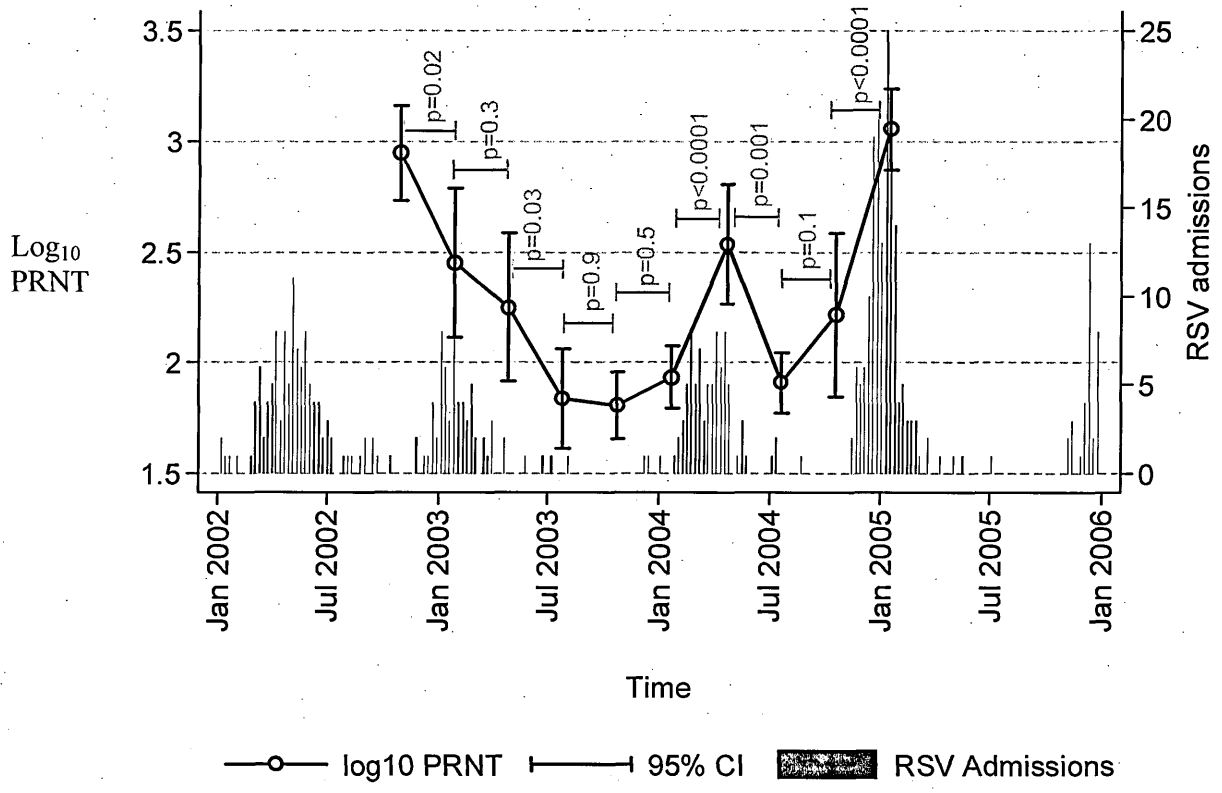

Figure 6.4 The temporal dynamics of the neutralising response in cohort 2 relative to RSV occurrence as defined through hospital admissions surveillance . Sera from 9 individuals who were prospectively recruited and followed up for approximately 30 months are included in this analysis.Ten strata of 3 months were defined and the mean neutralising titre in each stratum compared to that of the subsequent stratum. The titres used in this analysis were mean titres obtained using two local test viruses (Ken/A/2006 and Ken/B/2008). The numbers within each stratum are shown in table 6.3

Stratum	First date	Last Date	Mean age - Months (range)	n	% within epidemic
1	12-Dec-02	24-Jan-03	0 (0-0)	5	100
2	29-Jan-03	14-Apr-03	1.4 (0-2.9)	6	100
3	07-May-03	18-Jul-03	3.9 (0-6.9)	8	0
4	05-Aug-03	15-Oct-03	7.1 (3-9.8)	7	0
5	03-Nov-03	19-Jan-04	9.3 (5.8-11)	6	17
6	23-Jan-04	19-Apr-04	13.3 (9.1-15.8)	14	100
7	21-Apr-04	16-Jul-04	15 (11.1-18.1)	15	67
8	27-Jul-04	15-Sep-04	18.1 (15-20.2)	5	0
9	25-Oct-04	07-Jan-05	21.2 (18.1-24.8)	10	30
10	18-Jan-05	26-Feb-05	24.6 (21.1-26.5)	7	71

Table 6.3 The start and end dates, mean age in months, total number of samples and proportion of samples collected during an epidemic for each cohort 2 stratum shown in figure 6.4.

The ability to maintain high neutralising antibody titres after infection was evaluated by comparing mean neutralising responses during epidemics to mean neutralising responses during inter-epidemic periods among infants who had experienced a primary infection. These analyses only included samples collected from infants who were three months or older to reduce the effect of maternal antibody titres on the calculated estimates. The results of multiple regression analysis showed that the mean neutralising antibody titres during epidemics (1.9 $\log_{10}$ PRNT) was significantly greater than the mean neutralising titres during inter-epidemic periods (1.6 $\log_{10}$ PRNT, p=0.001). These data are graphically presented in figure 6.5.

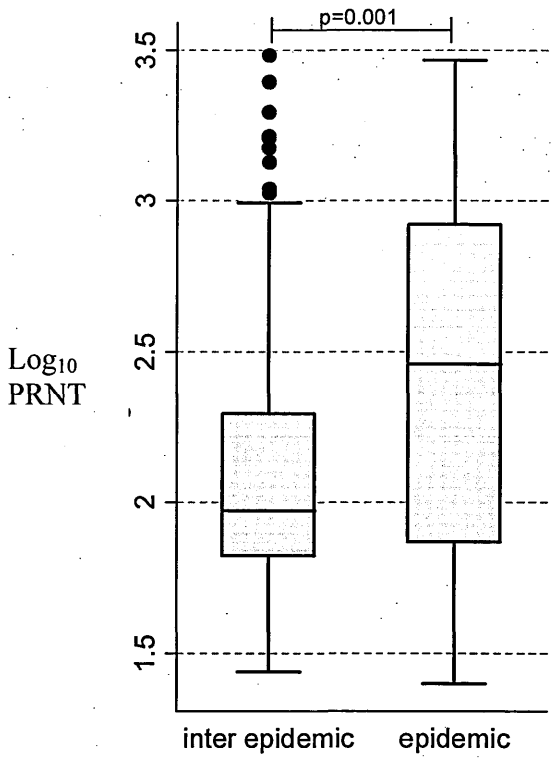

Figure 6.5 The distribution of neutralising antibody titres during epidemic and inter-epidemic periods for an infant birth cohort from coastal Kenya.

The duration of the primary neutralising response

In order to calculate the duration of the primary neutralising antibody response, antibody titres at different time points post-infection were compared to the pre-exposure control (figure 6.6). Results from multiple linear regression analysis showed that there was no difference between neutralising titres in the pre-exposure control group (1.8 $\log_{10}$ PRNT) and in sera collected between 0-0.4 months after infection (1.9 $\log_{10}$ PRNT, p=0.146). There was however a significant difference between neutralising titres in the pre-exposure control and at 0.5–0.9 months post infection (2.8 $\log_{10}$ PRNT, p<0.0001), 1.0-1.9 months post infection (2.5 $\log_{10}$ PRNT, p<0.0001) and at 2.0 - 2.9 months post infection (2.3 $\log_{10}$ PRNT, p<0.0001).

There was no difference between neutralising titres in the pre-exposure control and neutralising titres at 3.0-3.9 months post infection (2.0 $\log_{10}$ PRNT, p=0.052). These data are graphically shown in figure 6.6.

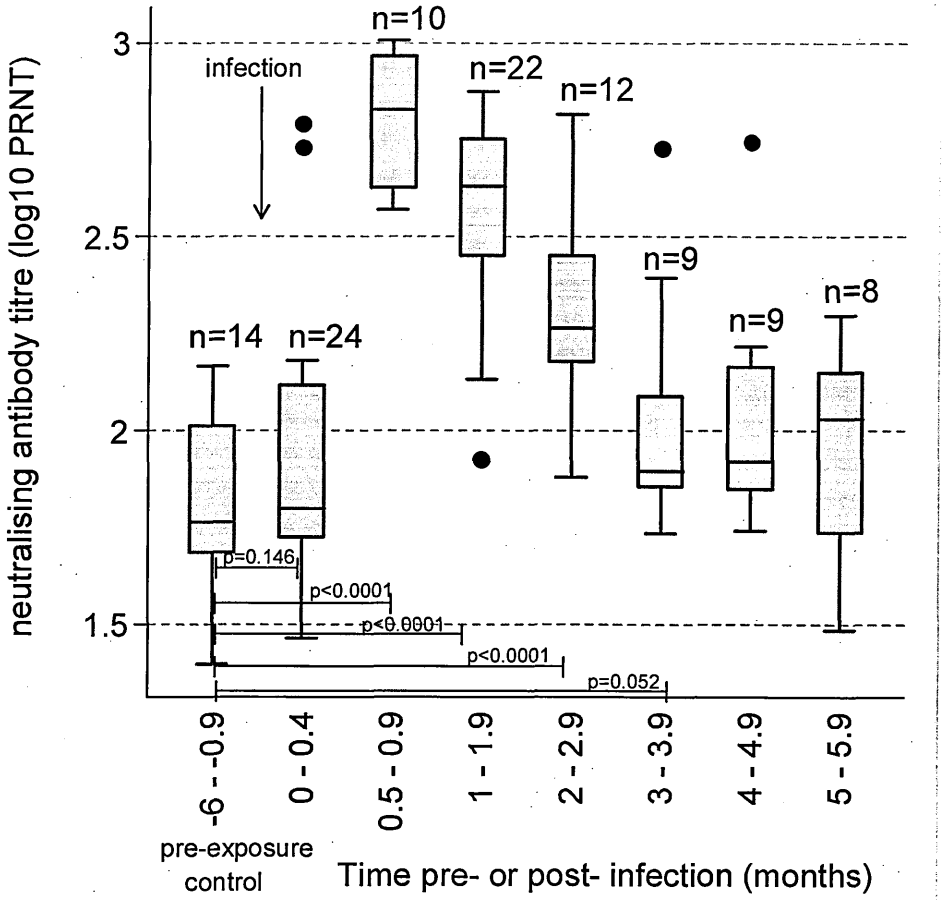

Figure 6.6 The duration of the primary neutralising antibody response. The mean neutralising titres at various time points post infection were compared to the mean titre of a pre-exposure control group. The p values indicate whether there is a significant difference between post infection titres at different time points post infection and the pre-exposure control. The number of samples within each time point are shown above each respective distribution.

Duration of protective, maternally-derived neutralising antibodies

Neutralising antibody titres measured in sera obtained prior to the identification of a primary infection were classified into 2 levels: high and low using a threshold titre (2.48 $\log_{10}$ PRNT) that has been associated with protection from severe disease in infants (Glezen *et al.*, 1981a). Age specific prevalence estimates of maternal neutralising antibody titres above this threshold were then determined. At between 0 and 0.9 months of age 100% (95% CI 86%-100%) of infants had titres above the protective threshold. This proportion dropped to 37% (95% CI 16%-62%) at between 2 and 2.9 months of age. No infants between 3 and 4.9 months of age had maternal neutralising antibodies above the protective threshold. These data are graphically presented in figure 6.7. Using survival analysis, it was estimated that mean duration of protective maternal neutralising antibodies is 2.8 months (95% CI 2.5 – 3.1 months).

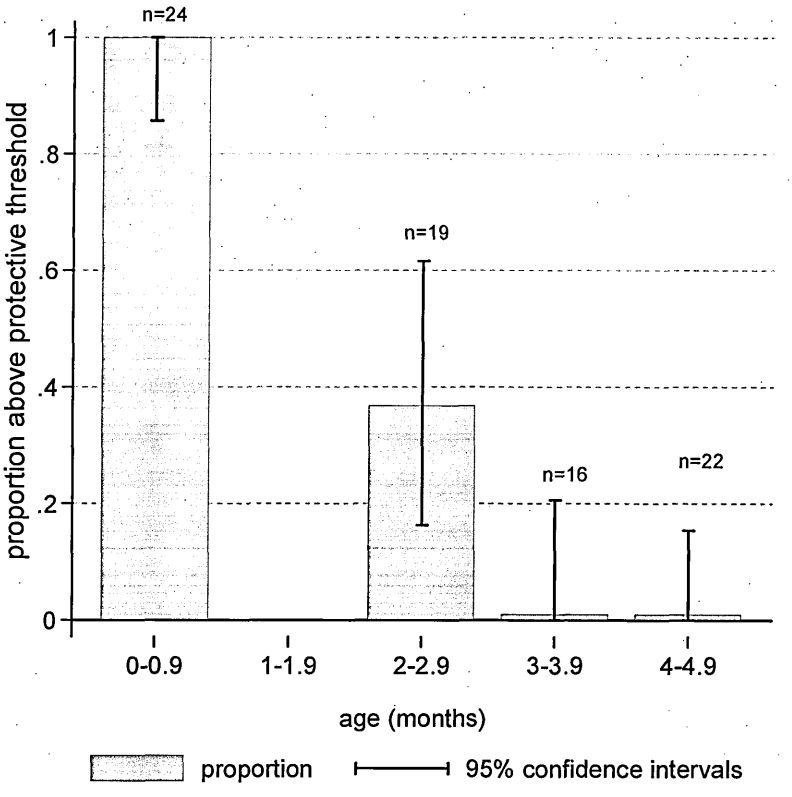

Figure 6.7 The proportion of infants in different age classes with mean neutralising titres that are above a putative protective threshold (Glezen *et al.*, 1981a). The bars indicate the proportion of samples that were above the putative protective threshold, while the lines traversing the bars indicate 95% confidence intervals. No data were available for the 1-1.9 month age class. The number of infants within each age class is shown above the proportion bars.

The rate of development of neutralising antibodies following primary and secondary infection

Neutralising titres measured in sera collected within 10 days of identification of primary and secondary infection by IFAT were compared. There was no difference between the mean neutralising antibody titre of the pre exposure control and the mean neutralising titre in the sera of primary infected infants collected with 10 days of identification of the infection (1.9 $\log_{10}$ PRNT, p=0.448). However, the mean neutralising titre in the sera collected within 10 days of identification of secondary

infection (3.02 $\log_{10}$ PRNT) was significantly greater than the mean response in the pre exposure control ($p<0.0001$) and the mean response in the sera of primary infected infants ($p<0.0001$). These data are graphically presented in figure 6.8. Mean convalescent responses to both primary and secondary infection were also compared.

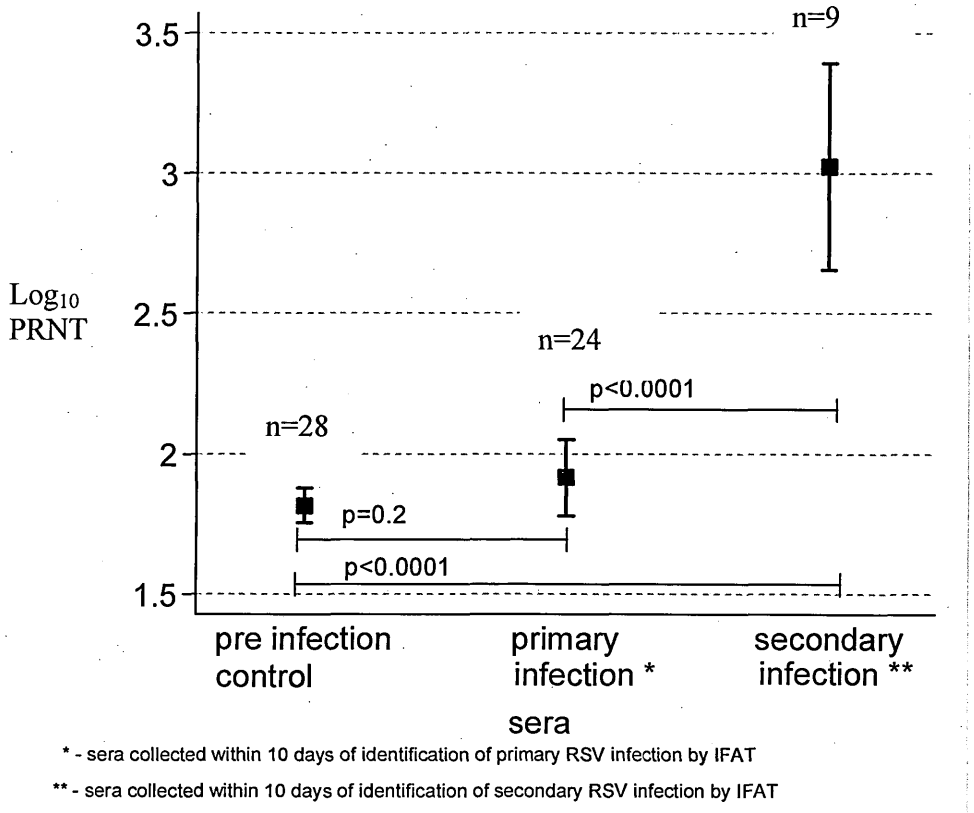

Figure 6.8 Neutralising titres in sera collected within 10 days of identification of RSV during primary and secondary infection are compared to a pre-exposure control. The dark boxes indicate the mean titre within each group while the whiskers indicate 95% confidence intervals. The p values indicate differences in mean titre between the comparison groups are statistically significant. The number of samples within each group is indicated above the mean and 95% confidence interval symbols.

Discussion

This chapter looked at the kinetics of the RSV neutralising response following natural infection. The data presented in this study was obtained by measuring the serum neutralising antibody responses in 2 RSV birth cohorts, which were prospectively followed up for up to 30 months or until infants had experienced at least 3 epidemics.

In the first epidemic experienced by either cohort, there was a general decline of neutralising antibody, despite indirect evidence of exposure. This lack of response is most likely related to the presence of maternally-derived neutralising antibodies that are typically present in relatively high titres in the first few months of life. It is possible that these antibodies provided some level of protection from natural infection, or masked the infants' native response to infection. This masking of infant responses early in life by maternally-derived antibodies has been demonstrated in earlier studies (Parrott *et al.*, 1973). The second epidemic occurred at a time when most infants within both cohorts were at an age in which maternal antibodies had declined to very low levels. The data show that at the time infants in both cohorts were experiencing the second RSV epidemic of their lives, mean neutralising antibodies within both cohorts rose in tandem with community level transmission of RSV and declined with the waning of population level transmission. These data strongly suggest that community level exposure in the form of RSV epidemics, triggers herd neutralising responses among infants in whom maternally-derived antibody has declined to minimal levels and that these responses are short-lived. Analysis of the duration of the primary neutralising response showed that it declined to pre-infection levels within 3-4 months post infection. Taken together, these data suggest that susceptibility to secondary may be established in part as a result of the

rapid decline of primary neutralising antibodies. Glezen *et al.* showed that up to 76% of infants who experience primary infection in their first year of life, go on to experience secondary infection in their second year of life (Glezen *et al.*, 1986), suggesting that immunity generated following primary infection is not sufficient to protect from natural challenge in the subsequent RSV epidemic.

The study by Glezen *et al.* showed that the risk of re-infection was inversely related to the titre of pre-existing neutralising antibody, suggesting that neutralising antibodies are associated with protection from infection and disease. This idea has been supported by studies of experimental adult infection (Hall *et al.*, 1991, Lee *et al.*, 2004), in which the level of pre-inoculation neutralising titres have been found to be negatively associated with susceptibility to experimental infection. The data presented in this study suggests that future RSV vaccines may have to be repeatedly administered to boost waning protective antibodies among young infants if the goal is the prevention of RSV infection and subsequent spread. The data presented in this study support previous suggestions of the transient nature of antibody-mediated immunity to primary RSV infection. Previous studies have found that the total infant serum IgG, IgM and IgA antibody response to primary RSV infection, declined to pre-infection levels within one year (Welliver *et al.*, 1981) while the secretory IgA response declined to pre-infection levels within 3 months post infection (Kaul *et al.*, 1981). Among adults, serum neutralising responses have been found to decline significantly within the one year after natural infection (Falsey *et al.*, 2006). The data presented in this study support the notion that primary immune responses to RSV are short-lived and may be related to susceptibility for secondary infection. The role of strain variation in facilitating re-infection is not yet clear. Despite evidence that re-

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APPENDIX 1

```
. //CHAPTER 5
.
. ////////////////////////////////////////////////////////////////////////////
> // This code compares mean acute phase antibodies at different ages using
. //  a logistic regression model
. ////////////////////////////////////////////////////////////////////////////
>
. egen ag6=cut  (age_m),at  (0,1,2,3,4,5,6,12,99)
  (7 missing values generated)

. xi:regress  log10acute ib0.ag6

      Source |       SS       df       MS              Number of obs =     286
-------------+------------------------------           F  (  7,   278) =   16.87
       Model |  9.24905608     7  1.32129373           Prob > F      =  0.0000
    Residual |  21.7776358   278  .078336819           R-squared     =  0.2981
-------------+------------------------------           Adj R-squared =  0.2804
       Total |  31.0266919   285  .108865586           Root MSE      =  .27989

------------------------------------------------------------------------------
  log10acute |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         ag6 |
          1  |  -.1358141   .0711284    -1.91   0.057    -.2758328    .0042046
          2  |  -.2234678   .0675665    -3.31   0.001    -.3564748   -.0904607
          3  |  -.3156109   .0896856    -3.52   0.001    -.4921602   -.1390617
          4  |  -.5225759   .0731395    -7.14   0.000    -.6665535   -.3785983
          5  |  -.3920144   .0786025    -4.99   0.000     -.546746   -.2372827
          6  |  -.5562053    .061267    -9.08   0.000    -.6768114   -.4355992
         12  |  -.3621963   .0604418    -5.99   0.000     -.481178   -.2432146
             |
       _cons |   2.660786   .0494775    53.78   0.000     2.563387    2.758184
------------------------------------------------------------------------------

. xi:regress  log10acute ib1.ag6

      Source |       SS       df       MS              Number of obs =     286
-------------+------------------------------           F  (  7,   278) =   16.87
       Model |  9.24905608     7  1.32129373           Prob > F      =  0.0000
    Residual |  21.7776358   278  .078336819           R-squared     =  0.2981
-------------+------------------------------           Adj R-squared =  0.2804
       Total |  31.0266919   285  .108865586           Root MSE      =  .27989

------------------------------------------------------------------------------
  log10acute |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         ag6 |
```

```
           0  |   .1358141   .0711284     1.91   0.057    -.0042046    .2758328
           2  |  -.0876537   .0687636    -1.27   0.203    -.2230172    .0477099
           3  |  -.1797968   .0905909    -1.98   0.048    -.3581281   -.0014655
           4  |  -.3867618   .0742468    -5.21   0.000    -.5329192   -.2406044
           5  |  -.2562003   .0796339    -3.22   0.001    -.4129622   -.0994383
           6  |  -.4203912   .0625847    -6.72   0.000    -.5435913   -.2971912
          12  |  -.2263822   .0617771    -3.66   0.000    -.3479925   -.1047719
              |
       _cons  |   2.524971   .0511002    49.41   0.000     2.424379    2.625564
------------------------------------------------------------------------------

. xi:regress  log10acute ib2.ag6

      Source |       SS       df       MS              Number of obs =     286
-------------+------------------------------           F (  7,   278) =   16.87
       Model |  9.24905608     7  1.32129373           Prob > F      =  0.0000
    Residual |  21.7776358   278  .078336819           R-squared     =  0.2981
-------------+------------------------------           Adj R-squared =  0.2804
       Total |  31.0266919   285  .108865586           Root MSE      =  .27989

------------------------------------------------------------------------------
  log10acute |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         ag6 |
           0 |   .2234678   .0675665     3.31   0.001     .0904607    .3564748
           1 |   .0876537   .0687636     1.27   0.203    -.0477099    .2230172
           3 |  -.0921432    .087822    -1.05   0.295    -.2650237    .0807374
           4 |  -.2991081   .0708419    -4.22   0.000    -.4385628   -.1596534
           5 |  -.1685466   .0764692    -2.20   0.028    -.3190788   -.0180144
           6 |  -.3327375   .0585049    -5.69   0.000    -.4479065   -.2175686
          12 |  -.1387285   .0576402    -2.41   0.017    -.2521952   -.0252618
             |
       _cons |   2.437318   .0460132    52.97   0.000     2.346739    2.527896
------------------------------------------------------------------------------

. xi:regress  log10acute ib3.ag6

      Source |       SS       df       MS              Number of obs =     286
-------------+------------------------------           F (  7,   278) =   16.87
       Model |  9.24905608     7  1.32129373           Prob > F      =  0.0000
    Residual |  21.7776358   278  .078336819           R-squared     =  0.2981
-------------+------------------------------           Adj R-squared =  0.2804
       Total |  31.0266919   285  .108865586           Root MSE      =  .27989

------------------------------------------------------------------------------
  log10acute |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         ag6 |
           0 |   .3156109   .0896856     3.52   0.001     .1390617    .4921602
           1 |   .1797968   .0905909     1.98   0.048     .0014655    .3581281
           2 |   .0921432    .087822     1.05   0.295    -.0807374    .2650237
```

```
           4  |   -.206965   .0921784    -2.25   0.026    -.3884212   -.0255087
           5  |  -.0764034   .0965702    -0.79   0.430    -.2665052    .1136984
           6  |  -.2405944   .0830729    -2.90   0.004    -.4041261   -.0770626
          12  |  -.0465854   .0824662    -0.56   0.573    -.2089228    .1157521
              |
       _cons  |   2.345175    .074803    31.35   0.000     2.197922    2.492427
------------------------------------------------------------------------------

. xi:regress  log10acute ib4.ag6

      Source |       SS       df       MS              Number of obs =     286
-------------+------------------------------           F(  7,   278) =   16.87
       Model |  9.24905608     7  1.32129373           Prob > F      =  0.0000
    Residual |  21.7776358   278  .078336819           R-squared     =  0.2981
-------------+------------------------------           Adj R-squared =  0.2804
       Total |  31.0266919   285  .108865586           Root MSE      =  .27989

------------------------------------------------------------------------------
  log10acute |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
        ag6 |
           0  |   .5225759   .0731395     7.14   0.000     .3785983    .6665535
           1  |   .3867618   .0742468     5.21   0.000     .2406044    .5329192
           2  |   .2991081   .0708419     4.22   0.000     .1596534    .4385628
           3  |    .206965   .0921784     2.25   0.026     .0255087    .3884212
           5  |   .1305615   .0814352     1.60   0.110    -.0297464    .2908694
           6  |  -.0336294   .0648612    -0.52   0.605    -.1613109    .0940521
          12  |   .1603796   .0640823     2.50   0.013     .0342314    .2865279
              |
       _cons  |    2.13821   .0538643    39.70   0.000     2.032176    2.244243
------------------------------------------------------------------------------

. xi:regress  log10acute ib5.ag6

      Source |       SS       df       MS              Number of obs =     286
-------------+------------------------------           F(  7,   278) =   16.87
       Model |  9.24905608     7  1.32129373           Prob > F      =  0.0000
    Residual |  21.7776358   278  .078336819           R-squared     =  0.2981
-------------+------------------------------           Adj R-squared =  0.2804
       Total |  31.0266919   285  .108865586           Root MSE      =  .27989

------------------------------------------------------------------------------
  log10acute |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
        ag6 |
           0  |   .3920144   .0786025     4.99   0.000     .2372827     .546746
           1  |   .2562003   .0796339     3.22   0.001     .0994383    .4129622
           2  |   .1685466   .0764692     2.20   0.028     .0180144    .3190788
           3  |   .0764034   .0965702     0.79   0.430    -.1136984    .2665052
           4  |  -.1305615   .0814352    -1.60   0.110    -.2908694    .0297464
           6  |  -.1641909   .0709643    -2.31   0.021    -.3038867   -.0244952
```

```
         12  |   .0298181   .0702532     0.42   0.672    -.1084777    .1681138
             |
       _cons |   2.268771   .0610764    37.15   0.000      2.14854    2.389002
------------------------------------------------------------------------------

. xi:regress  log10acute ib6.ag6

      Source |       SS       df       MS              Number of obs =     286
-------------+------------------------------           F  (  7,   278) =   16.87
       Model |  9.24905608     7  1.32129373           Prob > F      =  0.0000
    Residual |  21.7776358   278  .078336819           R-squared     =  0.2981
-------------+------------------------------           Adj R-squared =  0.2804
       Total |  31.0266919   285  .108865586           Root MSE      =  .27989

------------------------------------------------------------------------------
  log10acute |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         ag6 |
          0  |   .5562053    .061267     9.08   0.000     .4355992    .6768114
          1  |   .4203912   .0625847     6.72   0.000     .2971912    .5435913
          2  |   .3327375   .0585049     5.69   0.000     .2175686    .4479065
          3  |   .2405944   .0830729     2.90   0.004     .0770626    .4041261
          4  |   .0336294   .0648612     0.52   0.605    -.0940521    .1613109
          5  |   .1641909   .0709643     2.31   0.021     .0244952    .3038867
         12  |    .194009   .0501078     3.87   0.000       .09537     .292648
             |
       _cons |    2.10458   .0361333    58.24   0.000     2.033451     2.17571
------------------------------------------------------------------------------


. ////////////////////////////////////////////////////////////////////////////
> // This code compares mean acute and convalescent phase titres using a
. // student t test
. ////////////////////////////////////////////////////////////////////////////
> //analysis of diff between acute and con in diff age classes
. ttest   log10acute== log10conv  if ag6==0

Paired t test
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
log10a~e |      28    2.665101    .0388957    .2058165    2.585293    2.744908
log10c~v |      28    2.543671    .0535186    .2831937     2.43386    2.653482
---------+--------------------------------------------------------------------
    diff |      28    .1214302     .069361    .3670239   -.0208869    .2637472
------------------------------------------------------------------------------
     mean  (diff) = mean  (log10acute - log10conv)                    t =   1.7507
 Ho: mean  (diff) = 0                              degrees of freedom =       27

 Ha: mean  (diff) < 0          Ha: mean  (diff) != 0          Ha: mean  (diff) > 0
```

```
 Pr  (T < t) = 0.9543         Pr  (|T| > |t|) = 0.0914          Pr  (T > t) = 0.0457

. ttest  log10acute== log10conv  if ag6==1

Paired t test
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
log10a~e |      25    2.572685    .0481229    .2406144    2.473365    2.672006
log10c~v |      25    2.473255    .0739728    .3698642    2.320583    2.625928
---------+--------------------------------------------------------------------
    diff |      25    .0994304    .0958033    .4790166   -.0982979    .2971587
------------------------------------------------------------------------------
     mean  (diff) = mean  (log10acute - log10conv)                    t =   1.0379
 Ho: mean  (diff) = 0                                 degrees of freedom =       24

 Ha: mean  (diff) < 0           Ha: mean  (diff) != 0           Ha: mean  (diff) > 0
 Pr  (T < t) = 0.8452         Pr  (|T| > |t|) = 0.3097          Pr  (T > t) = 0.1548

. ttest  log10acute== log10conv  if ag6==2

Paired t test
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
log10a~e |      34    2.456123     .032705    .1907012    2.389585    2.522662
log10c~v |      34    2.566452     .062091    .3620494    2.440127    2.692777
---------+--------------------------------------------------------------------
    diff |      34   -.1103288    .0813282     .474221   -.2757924    .0551347
------------------------------------------------------------------------------
     mean  (diff) = mean  (log10acute - log10conv)                    t =  -1.3566
 Ho: mean  (diff) = 0                                 degrees of freedom =       33

 Ha: mean  (diff) < 0           Ha: mean  (diff) != 0           Ha: mean  (diff) > 0
 Pr  (T < t) = 0.0921         Pr  (|T| > |t|) = 0.1841          Pr  (T > t) = 0.9079

. ttest  log10acute== log10conv  if ag6==3

Paired t test
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
log10a~e |      11    2.437465    .0599942    .1989782    2.303789     2.57114
log10c~v |      11    2.492986    .1130327    .3748871    2.241133    2.744838
---------+--------------------------------------------------------------------
    diff |      11    -.055521    .1439012    .4772663   -.3761528    .2651109
------------------------------------------------------------------------------
     mean  (diff) = mean  (log10acute - log10conv)                    t =  -0.3858
 Ho: mean  (diff) = 0                                 degrees of freedom =       10

 Ha: mean  (diff) < 0           Ha: mean  (diff) != 0           Ha: mean  (diff) > 0
```

```
 Pr  (T < t) = 0.3539         Pr  (|T| > |t|) = 0.6077          Pr  (T > t) = 0.6461

. ttest  log10acute== log10conv  if ag6==4

Paired t test
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
log10a~e |      19    2.198126    .0545468    .2377641    2.083528    2.312725
log10c~v |      19    2.502542    .0827452    .3606781    2.328701    2.676383
---------+--------------------------------------------------------------------
    diff |      19   -.3044157     .114603    .4995428   -.5451876   -.0636438
------------------------------------------------------------------------------
     mean  (diff) = mean  (log10acute - log10conv)                    t =  -2.6563
 Ho: mean  (diff) = 0                                 degrees of freedom =       18

 Ha: mean  (diff) < 0          Ha: mean  (diff) != 0          Ha: mean  (diff) > 0
 Pr  (T < t) = 0.0080        Pr  (|T| > |t|) = 0.0161         Pr  (T > t) = 0.9920

. ttest  log10acute== log10conv  if ag6==5

Paired t test
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
log10a~e |      19    2.292477    .0639136    .2785929    2.158199    2.426754
log10c~v |      19    2.647895    .1004128    .4376893    2.436935    2.858854
---------+--------------------------------------------------------------------
    diff |      19   -.3554184    .1279322    .5576434   -.6241939   -.0866429
------------------------------------------------------------------------------
     mean  (diff) = mean  (log10acute - log10conv)                    t =  -2.7782
 Ho: mean  (diff) = 0                                 degrees of freedom =       18

 Ha: mean  (diff) < 0          Ha: mean  (diff) != 0          Ha: mean  (diff) > 0
 Pr  (T < t) = 0.0062        Pr  (|T| > |t|) = 0.0124         Pr  (T > t) = 0.9938

. ttest  log10acute== log10conv  if ag6==6

Paired t test
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
log10a~e |      58    2.125542    .0329551    .2509785     2.05955    2.191533
log10c~v |      58    2.767628    .0386969    .2947069    2.690139    2.845117
---------+--------------------------------------------------------------------
    diff |      58   -.6420865    .0519842    .3959001   -.7461831   -.5379899
------------------------------------------------------------------------------
     mean  (diff) = mean  (log10acute - log10conv)                    t = -12.3516
 Ho: mean  (diff) = 0                                 degrees of freedom =       57

 Ha: mean  (diff) < 0          Ha: mean  (diff) != 0          Ha: mean  (diff) > 0
```

```
 Pr  (T < t) = 0.0000         Pr  (|T| > |t|) = 0.0000          Pr  (T > t) = 1.0000

. ttest   log10acute== log10conv  if ag6==12

Paired t test
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
log10a~e |      60    2.304789    .0461592    .3575475    2.212425    2.397154
log10c~v |      60    2.814946    .0454438    .3520061    2.724013    2.905879
---------+--------------------------------------------------------------------
    diff |      60   -.5101565    .0591738    .4583585   -.6285631   -.3917499
------------------------------------------------------------------------------
     mean  (diff) = mean  (log10acute - log10conv)                    t =  -8.6213
 Ho: mean  (diff) = 0                                 degrees of freedom =       59

 Ha: mean  (diff) < 0           Ha: mean  (diff) != 0           Ha: mean  (diff) > 0
 Pr  (T < t) = 0.0000         Pr  (|T| > |t|) = 0.0000          Pr  (T > t) = 1.0000

.
end of do-file


. ////////////////////////////////////////////////////////////////////////////
> // This code conducts spearman correlation analysis between log fold rise in titre
. // and  (i) acute phase titres and  (ii) age in months
. ////////////////////////////////////////////////////////////////////////////
>
. spearman    log10acute log10foldrise

 Number of obs =     254
Spearman's rho =      -0.7451

Test of Ho: log10acute and log10foldrise are independent
    Prob > |t| =       0.0000

. spearman    log10foldrise age_m  if age_m<=12

 Number of obs =     194
Spearman's rho =       0.5511

Test of Ho: log10foldrise and age_m are independent
    Prob > |t| =       0.0000

.
end of do-file


. ////////////////////////////////////////////////////////////////////////////
> // This code calculates the proportion seroconverting at different acute stage
```

```
. // titres stratified by quartile
. ////////////////////////////////////////////////////////////////////////////
>
. tab fold4  acute_quartiles if i_gp==t_gp,col

+-------------------+
| Key               |
|-------------------|
|     frequency     |
| column percentage |
+-------------------+

           |               acute_quartiles
     fold4 |         1          2          3          4 |     Total
-----------+--------------------------------------------+----------
         0 |         4         19         32         26 |        81
           |      9.76      40.43      86.49     100.00 |     53.64
-----------+--------------------------------------------+----------
         1 |        37         28          5          0 |        70
           |     90.24      59.57      13.51       0.00 |     46.36
-----------+--------------------------------------------+----------
     Total |        41         47         37         26 |       151
           |    100.00     100.00     100.00     100.00 |    100.00
```

```
. ////////////////////////////////////////////////////////////////////////////
> // This code calculates the odds ratios of developing different features of
. // clinical pneumonia among infants with titres above and below the 2.5log10PRNT
. // threshold
. ////////////////////////////////////////////////////////////////////////////
>                 preserve

.             use "C:\Documents and Settings\csande.KWTRP\Desktop\paper1\IP_clinical
severity data.d
> ta", clear

.                 capture: drop prot_acute

.                 gen prot_acute=.
  (322 missing values generated)

.                 replace prot_acute=1 if log10acute>=2.5 & log10acute!=.
  (85 real changes made)
```

```
.                 replace prot_acute=0 if log10acute<2.5  & log10acute!=.
  (201 real changes made)

.
.
.                 xi:logistic    prot_acute  i.flaring i.wheeze  i.indrawing i.shock
i.hypoxia i.cra
> ckles i.ag6  ,or
i.flaring         _Iflaring_0-1  (naturally coded; _Iflaring_0 omitted)
i.wheeze          _Iwheeze_0-1  (naturally coded; _Iwheeze_0 omitted)
i.indrawing       _Iindrawing_0-1  (naturally coded; _Iindrawing_0 omitted)
i.shock           _Ishock_0-1  (naturally coded; _Ishock_0 omitted)
i.hypoxia         _Ihypoxia_0-1  (naturally coded; _Ihypoxia_0 omitted)
i.crackles        _Icrackles_0-1  (naturally coded; _Icrackles_0 omitted)
i.ag6             _Iag6_0-24  (naturally coded; _Iag6_0 omitted)
note: _Iag6_9 != 0 predicts failure perfectly
      _Iag6_9 dropped and 22 obs not used


Logistic regression                               Number of obs   =        232
                                                  LR chi2  (15)     =      53.46
                                                  Prob > chi2     =     0.0000
Log likelihood = -120.00178                       Pseudo R2       =     0.1822

------------------------------------------------------------------------------
  prot_acute | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
 _Iflaring_1 |   .6577176   .2501528    -1.10   0.271      .312103    1.386056
  _Iwheeze_1 |    .966315   .4581894    -0.07   0.942     .3815151    2.447517
_Iindrawin~1 |    .820654   .6201792    -0.26   0.794     .1865931    3.609313
   _Ishock_1 |   .2656081   .1461305    -2.41   0.016      .090351    .7808177
 _Ihypoxia_1 |   .7422788   .4326905    -0.51   0.609     .2368008    2.326757
_Icrackles_1 |   .8088687   .2992585    -0.57   0.566     .3917036    1.670316
     _Iag6_1 |   .2940198   .1737413    -2.07   0.038     .0923394    .9361944
     _Iag6_2 |   .2933712    .173697    -2.07   0.038     .0919273    .9362472
     _Iag6_3 |   .1183904    .091972    -2.75   0.006      .025826    .5427197
     _Iag6_4 |   .0109983   .0126067    -3.93   0.000     .0011632    .1039937
     _Iag6_5 |    .078147   .0653201    -3.05   0.002     .0151853    .4021607
     _Iag6_6 |   .0312443   .0246607    -4.39   0.000     .0066516    .1467614
     _Iag6_9 |  (omitted)
    _Iag6_12 |   .1313523   .1138732    -2.34   0.019     .0240163    .7184055
    _Iag6_18 |   .1291534   .0859975    -3.07   0.002      .035021    .4763028
    _Iag6_24 |   .1164771   .0803751    -3.12   0.002     .0301209    .4504148
------------------------------------------------------------------------------

.
.                 parmest, norestore eform

.                 drop if parm=="_cons"
  (1 observation deleted)
```

```
. drop if parm=="age_months"
  (0 observations deleted)

. drop if parm== "_Iag6_1"
  (1 observation deleted)

. drop if parm== "_Iag6_2"
  (1 observation deleted)

. drop if parm== "_Iag6_3"
  (1 observation deleted)

. drop if parm== "_Iag6_4"
  (1 observation deleted)

. drop if parm== "_Iag6_5"
  (1 observation deleted)

. drop if parm== "_Iag6_6"
  (1 observation deleted)

. drop if parm== "_Iag6_9"
  (0 observations deleted)

. drop if parm== "_Iag6_12"
  (1 observation deleted)

. drop if parm== "_Iag6_18"
  (1 observation deleted)

. drop if parm== "_Iag6_24"
  (1 observation deleted)

. drop if parm== "o._Iag6_9"
  (1 observation deleted)

.
. egen axis= axis  (estimate), label  (parm)

. twoway scatter  axis estimate, xline  (1) ylab  (, valuelabel angle
(0)) ||  rcap min9
> 5 max95 axis, horizontal legend  (order  (1 "point estimate"  2 "95% conf. int.")
pos  (6)) xtitle  ("odds
>  ratio") ytitle  ("")

. graph play "OR_2"

.
. restore
```

```
. ////////////////////////////////////////////////////////////////////////////
> // This code compares the proportions seroconverting to different virus
. ////////////////////////////////////////////////////////////////////////////
>
.
. // comparison between no infected with RSV A seroconverting against Kil/A/2006 &
Kil/B/2008
. preserve

. keep   if   a_match==2 & foldrise!=. | a_match==1 & i_gp==1 & foldrise!=.
  (258 observations deleted)

. keep serial_no t_virus_desc fold4

. sort serial_no t_virus_desc

. reshape wide fold4, i  (serial_no) j  (t_virus_desc)
  (note: j = 2 4)

Data                               long   ->   wide
-----------------------------------------------------------------------------
Number of obs.                       64   ->      32
Number of variables                   3   ->       3
j variable  (2 values)      t_virus_desc   ->  (dropped)
xij variables:
                                   fold4   ->   fold42 fold44
-----------------------------------------------------------------------------

. mcc fold42 fold44

                | Controls               |
Cases           |   Exposed   Unexposed  |      Total
----------------+------------------------+------------
         Exposed |         4          12  |         16
       Unexposed |         0          16  |         16
----------------+------------------------+------------
           Total |         4          28  |         32

McNemar's chi2  (1) =     12.00    Prob > chi2 = 0.0005
Exact McNemar significance probability       = 0.0005

Proportion with factor
       Cases             .5
       Controls        .125     [95% Conf. Interval]
                  ---------     --------------------
       difference      .375       .176013    .573987
       ratio              4      1.711902   9.346329
       rel. diff.  .4285714       .2452718   .6118711

       odds ratio         .      2.778597          .  (exact)
```

```
. restore

.
.
. // comparison between no infected with RSV B seroconverting against Kil/A/2006 &
Kil/B/2008
. preserve

. keep if   b_match==2 & foldrise!=. | b_match==1 & i_gp==2 & foldrise!=.
  (272 observations deleted)

. keep serial_no t_virus_desc fold4

. sort serial_no t_virus_desc

. reshape wide fold4, i  (serial_no) j  (t_virus_desc)
  (note: j = 2 4)

Data                               long   ->   wide
-----------------------------------------------------------------------------
Number of obs.                       50   ->      25
Number of variables                   3   ->       3
j variable  (2 values)      t_virus_desc   ->  (dropped)
xij variables:
                                  fold4   ->   fold42 fold44
-----------------------------------------------------------------------------

. mcc fold42 fold44

                | Controls               |
Cases           |   Exposed   Unexposed  |      Total
----------------+------------------------+-----------
        Exposed |         2           0  |          2
      Unexposed |         8          15  |         23
----------------+------------------------+-----------
          Total |        10          15  |         25

McNemar's chi2  (1) =      8.00    Prob > chi2 = 0.0047
Exact McNemar significance probability       = 0.0078

Proportion with factor
       Cases            .08
       Controls          .4     [95% Conf. Interval]
                  ---------     --------------------
       difference      -.32     -.5428553  -.0971447
       ratio             .2      .0579006   .6908394
       rel. diff. -.5333333     -.9909693  -.0756974

       odds ratio         0             0   .5858332  (exact)
```

```
. restore

.
. // comparison between no infected with RSV A seroconverting against A2 & 8/60
. preserve

. keep if   a_oldrsvs_match==2  |  a_oldrsvs_match==1 & i_gp==1
  (286 observations deleted)

. keep serial_no  t_virus_desc fold4

. sort serial_no t_virus_desc

. reshape wide fold4, i  (serial_no) j  (t_virus_desc)
  (note: j = 1 3)

Data                               long   ->   wide
-----------------------------------------------------------------------------
Number of obs.                       36   ->      18
Number of variables                   3   ->       3
j variable  (2 values)      t_virus_desc   ->  (dropped)
xij variables:
                                   fold4   ->   fold41 fold43
-----------------------------------------------------------------------------

. mcc fold41 fold43

                | Controls              |
Cases           |   Exposed   Unexposed |      Total
----------------+-----------------------+------------
        Exposed |         0           5 |          5
      Unexposed |         0          13 |         13
----------------+-----------------------+------------
          Total |         0          18 |         18

McNemar's chi2  (1) =      5.00    Prob > chi2 = 0.0253
Exact McNemar significance probability       = 0.0625

Proportion with factor
       Cases       .2777778
       Controls           0     [95% Conf. Interval]
                  ---------     --------------------
       difference  .2777778      .0153053   .5402503
       ratio             .             .          .
       rel. diff.  .2777778      .0708609   .4846947

       odds ratio         .      .9163559          .  (exact)

. restore

.
```

```
.
.
. // comparison between no infected with RSV B seroconverting against A2 & 8/60
. preserve

. keep if   b_oldrsvs_match==2 |  b_oldrsvs_match==1 & i_gp==2
  (282 observations deleted)

. keep serial_no  t_virus_desc fold4

. sort serial_no t_virus_desc

. reshape wide fold4, i  (serial_no) j  (t_virus_desc)
  (note: j = 1 3)

Data                               long   ->   wide
-----------------------------------------------------------------------------
Number of obs.                       40   ->      20
Number of variables                   3   ->       3
j variable  (2 values)      t_virus_desc   ->  (dropped)
xij variables:
                                   fold4   ->   fold41 fold43
-----------------------------------------------------------------------------

. mcc fold41 fold43

                | Controls               |
Cases           |   Exposed   Unexposed  |      Total
----------------+------------------------+-----------
         Exposed |         2           0  |          2
       Unexposed |        11           7  |         18
----------------+------------------------+-----------
           Total |        13           7  |         20

McNemar's chi2  (1) =     11.00    Prob > chi2 = 0.0009
Exact McNemar significance probability       = 0.0010

Proportion with factor
       Cases             .1
       Controls         .65     [95% Conf. Interval]
                  ---------     --------------------
       difference      -.55     -.8180322  -.2819678
       ratio       .1538462       .042996   .5504847
       rel. diff. -1.571429     -3.060562  -.0822951

       odds ratio         0             0   .3984335  (exact)

. restore
```

```
. ////////////////////////////////////////////////////////////////////////////
> // This code compares the magnitude of the homologous response to the magnitude
. // of the heterlogous response  (in terms of fold rise in titre) among different age
classes
. // using regression models
. ////////////////////////////////////////////////////////////////////////////
>
. //calculate fold rises for different matrix categories
. //antilog the table means to get the real fold rises
. tab i_gp t_gp if ag5==0,summ  (log10foldrise)means

                        Means of log10foldrise

Infecting_ |  Test_virus_group
 RSV_Group |         A          B |     Total
-----------+----------------------+----------
         A | .25803744 -.33280539 |-.02183548
         B |-.16332743  .42804353 | .21120751
-----------+----------------------+----------
     Total | .10852088  .05790081 | .08097761

. tab i_gp t_gp if ag5==6,summ  (log10foldrise)means

                        Means of log10foldrise

Infecting_ |  Test_virus_group
 RSV_Group |         A          B |     Total
-----------+----------------------+----------
         A | 1.0473335  .57577961 | .86596663
         B | .23170187   .7681455 | .57740999
-----------+----------------------+----------
     Total | .50357909   .7398564 | .64208648

. tab i_gp t_gp if ag5==12,summ  (log10foldrise)means

                        Means of log10foldrise

Infecting_ |  Test_virus_group
 RSV_Group |         A          B |     Total
-----------+----------------------+----------
         A | .85391161  .34706051 | .65117117
         B |-.02538991  .63216299 | .36914183
-----------+----------------------+----------
     Total |   .502191    .518122 |  .5101565


.
. //tid1 below contains matrix AA  (tid1==1) and AB  (tid1==2)
. //tid2 below contains matrix BB  (tid2==1) and BA  (tid2==2)
. capture: drop tid1 tid2
```

```
. gen tid1=.
  (322 missing values generated)

. gen tid2=.
  (322 missing values generated)

. replace tid1 =1  if i_gp==1 & t_gp==1
  (90 real changes made)

. replace tid1 =2  if i_gp==1 & t_gp==2
  (97 real changes made)

.
. replace tid2 =1  if i_gp==2 & t_gp==2
  (85 real changes made)

. replace tid2 =2  if i_gp==2 & t_gp==1
  (50 real changes made)

.
.
. // compare the fold rises between different hom het age classes
.
. xi: regress log10foldrise i.tid1 if  ag5==0
i.tid1            _Itid1_1-2  (naturally coded; _Itid1_1 omitted)

      Source |       SS       df       MS              Number of obs =      76
-------------+------------------------------           F  (  1,    74) =   45.65
       Model |  6.61443638     1  6.61443638           Prob > F      =  0.0000
    Residual |  10.7219889    74  .144891742           R-squared     =  0.3815
-------------+------------------------------           Adj R-squared =  0.3732
       Total |  17.3364253    75  .231152337           Root MSE      =  .38065

------------------------------------------------------------------------------
log10foldr~e |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Itid1_2 |  -.5908428   .0874475    -6.76   0.000    -.7650858   -.4165999
       _cons |   .2580374   .0601855     4.29   0.000     .1381152    .3779597
------------------------------------------------------------------------------

. xi: regress log10foldrise i.tid1 if  ag5==6
i.tid1            _Itid1_1-2  (naturally coded; _Itid1_1 omitted)

      Source |       SS       df       MS              Number of obs =      13
-------------+------------------------------           F  (  1,    11) =    7.45
       Model |   .68419411     1   .68419411           Prob > F      =  0.0196
    Residual |  1.00972921    11  .091793565           R-squared     =  0.4039
-------------+------------------------------           Adj R-squared =  0.3497
       Total |  1.69392332    12  .141160277           Root MSE      =  .30297

------------------------------------------------------------------------------
```

```
log10foldr~e |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Itid1_2 |  -.4715539   .1727221    -2.73   0.020    -.8517126   -.0913952
       _cons |   1.047334   .1071177     9.78   0.000     .8115691    1.283098
------------------------------------------------------------------------------

. xi: regress log10foldrise i.tid1 if  ag5==12
i.tid1            _Itid1_1-2  (naturally coded; _Itid1_1 omitted)

      Source |       SS       df       MS              Number of obs =      30
-------------+------------------------------           F  (  1,    28) =   14.95
       Model |  1.84966588     1  1.84966588           Prob > F      =  0.0006
    Residual |  3.46522804    28  .123758144           R-squared     =  0.3480
-------------+------------------------------           Adj R-squared =  0.3247
       Total |  5.31489393    29  .183272204           Root MSE      =  .35179

------------------------------------------------------------------------------
log10foldr~e |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Itid1_2 |  -.5068511   .1311054    -3.87   0.001    -.7754084   -.2382938
       _cons |   .8539116   .0829183    10.30   0.000     .6840611    1.023762
------------------------------------------------------------------------------

.
.
. xi: regress log10foldrise i.tid2 if  ag5==0
i.tid2            _Itid2_1-2  (naturally coded; _Itid2_1 omitted)

      Source |       SS       df       MS              Number of obs =      60
-------------+------------------------------           F  (  1,    58) =   30.46
       Model |  4.87275985     1  4.87275985           Prob > F      =  0.0000
    Residual |  9.27895563    58  .159981994           R-squared     =  0.3443
-------------+------------------------------           Adj R-squared =  0.3330
       Total |  14.1517155    59  .239859584           Root MSE      =  .39998

------------------------------------------------------------------------------
log10foldr~e |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Itid2_2 |   -.591371   .1071539    -5.52   0.000    -.8058628   -.3768791
       _cons |   .4280435   .0648849     6.60   0.000     .2981623    .5579248
------------------------------------------------------------------------------

. xi: regress log10foldrise i.tid2 if  ag5==6
i.tid2            _Itid2_1-2  (naturally coded; _Itid2_1 omitted)

      Source |       SS       df       MS              Number of obs =      45
-------------+------------------------------           F  (  1,    43) =   37.17
       Model |  2.96724671     1  2.96724671           Prob > F      =  0.0000
    Residual |  3.43300424    43  .079837308           R-squared     =  0.4636
-------------+------------------------------           Adj R-squared =  0.4511
       Total |  6.40025095    44  .145460249           Root MSE      =  .28255
```

```
------------------------------------------------------------------------------
log10foldr~e |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Itid2_2 |  -.5364436   .0879934    -6.10   0.000    -.7138993    -.358988
       _cons |   .7681455   .0524691    14.64   0.000     .6623314    .8739596
------------------------------------------------------------------------------

. xi: regress log10foldrise i.tid2 if  ag5==12
i.tid2            _Itid2_1-2  (naturally coded; _Itid2_1 omitted)

      Source |       SS       df       MS              Number of obs =      30
-------------+------------------------------           F(  1,    28) =   31.42
       Model |  3.11310586     1  3.11310586           Prob > F      =  0.0000
    Residual |  2.77435258    28  .099084021           R-squared     =  0.5288
-------------+------------------------------           Adj R-squared =  0.5119
       Total |  5.88745844    29  .203015808           Root MSE      =  .31478

------------------------------------------------------------------------------
log10foldr~e |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Itid2_2 |  -.6575529   .1173101    -5.61   0.000    -.8978518    -.417254
       _cons |    .632163   .0741934     8.52   0.000     .4801846    .7841414
------------------------------------------------------------------------------
```

APPENDIX 2

DATA ANALYSIS - STATA CODE

```
//=============================================================================
// Code: This code generates 3 monthly age classes for all the infants in the kinetics
//       study. The mean PRNT in each class is then calculated and compared to that of
//       the next age class.
//
//=============================================================================

. use "C:\Documents and
Settings\csande.KWTRP\Desktop\paper2\Full_COHORT_neut_surveillance overlay.dta",clear



. egen ag13=cut  (age_m), at  (0  (3)30)  //generate 3 monthly age classes
  (2046 missing values generated)

. collapse  (mean) l10=log10meannt  (sd) sd10=log10meannt  (count) cnt = log10meannt,by
(ag13) //calculate means, SDs and Ns for different age classes

. generate hi =  l10+ invttail  (cnt-1,0.025)*  (sd10 / sqrt  (cnt)) // calculate
upper CI
  (1 missing value generated)

. generate lo =  l10- invttail  (cnt-1,0.025)*  (sd10 / sqrt  (cnt)) // calculate
lower CI
  (1 missing value generated)

. capture: rename cohort cohort2

append using "C:\Documents and
Settings\csande.KWTRP\Desktop\paper2\Full_COHORT_neut_surveillance overlay.dta"
//merge with master file

.
. // comparison of PRNTs in different age classes
.
. xi:regress log10meannt ib0.ag13   // Base class --> 0-2 months of age

      Source |       SS       df       MS              Number of obs =     280
-------------+------------------------------           F  (  9,   270) =   15.28
       Model |   26.678699     9  2.96429989           Prob > F      =  0.0000
    Residual |  52.3688042   270  .193958534           R-squared     =  0.3375
-------------+------------------------------           Adj R-squared =  0.3154
```

```
       Total |  79.0475032   279  .283324384           Root MSE      =  .44041

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
        ag13 |
           3 |  -.5683188   .1010696    -5.62   0.000    -.7673036   -.3693341
           6 |  -.6753875   .1130249    -5.98   0.000    -.8979097   -.4528652
           9 |  -.4303978   .1000977    -4.30   0.000     -.627469   -.2333266
          12 |  -.3694693   .0966751    -3.82   0.000    -.5598021   -.1791364
          15 |  -.3983873   .1031822    -3.86   0.000    -.6015313   -.1952434
          18 |  -.5677476   .1148671    -4.94   0.000    -.7938967   -.3415984
          21 |  -.3110327   .1130249    -2.75   0.006    -.5335549   -.0885105
          24 |   .3375905   .1043333     3.24   0.001     .1321804    .5430007
          27 |   .2644401   .1528208     1.73   0.085    -.0364317     .565312
             |
       _cons |   2.607188   .0629153    41.44   0.000     2.483321    2.731055
------------------------------------------------------------------------------
```

. xi:regress log10meannt ib3.ag13 // Base class --> 3-5 months of age

```
      Source |       SS       df       MS              Number of obs =     280
-------------+------------------------------           F(  9,   270) =   15.28
       Model |  26.678699     9  2.96429989            Prob > F      =  0.0000
    Residual |  52.3688042   270  .193958534           R-squared     =  0.3375
-------------+------------------------------           Adj R-squared =  0.3154
       Total |  79.0475032   279  .283324384           Root MSE      =  .44041

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
        ag13 |
           0 |   .5683188   .1010696     5.62   0.000     .3693341    .7673036
           6 |  -.1070686   .1227722    -0.87   0.384    -.3487813     .134644
           9 |   .1379211   .1109862     1.24   0.215    -.0805873    .3564294
          12 |   .1988496   .1079095     1.84   0.066    -.0136015    .4113007
          15 |   .1699315   .1137759     1.49   0.136    -.0540692    .3939322
          18 |   .0005713   .1244703     0.00   0.996    -.2444844     .245627
          21 |   .2572861   .1227722     2.10   0.037     .0155735    .4989988
          24 |   .9059094   .1148208     7.89   0.000     .6798514    1.131967
          27 |    .832759   .1601642     5.20   0.000     .5174294    1.148089
             |
       _cons |    2.03887   .0790995    25.78   0.000     1.883139      2.1946
------------------------------------------------------------------------------
```

. xi:regress log10meannt ib6.ag13 // Base class --> 6-8 months of age

```
      Source |       SS       df       MS              Number of obs =     280
-------------+------------------------------           F(  9,   270) =   15.28
```

```
      Model |   26.678699     9  2.96429989           Prob > F      =  0.0000
   Residual |  52.3688042   270  .193958534           R-squared     =  0.3375
-------------+------------------------------           Adj R-squared =  0.3154
      Total |  79.0475032   279  .283324384           Root MSE      =  .44041

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
        ag13 |
           0 |   .6753875   .1130249     5.98   0.000     .4528652    .8979097
           3 |   .1070686   .1227722     0.87   0.384     -.134644    .3487813
           9 |   .2449897   .1219734     2.01   0.046     .0048499    .4851295
          12 |   .3059182   .1191807     2.57   0.011     .0712766    .5405598
          15 |   .2770001   .1245172     2.22   0.027     .0318521    .5221481
          18 |   .1076399   .1343593     0.80   0.424    -.1568852     .372165
          21 |   .3643548   .1327878     2.74   0.006     .1029236    .6257859
          24 |   1.012978   .1254727     8.07   0.000     .7659488    1.260007
          27 |   .9398276   .1679647     5.60   0.000     .6091405    1.270515
             |
       _cons |   1.931801   .0938951    20.57   0.000     1.746941    2.116661
------------------------------------------------------------------------------
```

. xi:regress log10meannt ib9.ag13 // Base class --> 9-11 months of age

```
      Source |       SS       df       MS              Number of obs =     280
-------------+------------------------------           F  (  9,   270) =   15.28
       Model |  26.678699     9  2.96429989           Prob > F      =  0.0000
    Residual |  52.3688042   270  .193958534           R-squared     =  0.3375
-------------+------------------------------           Adj R-squared =  0.3154
       Total |  79.0475032   279  .283324384           Root MSE      =  .44041

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
        ag13 |
           0 |   .4303978   .1000977     4.30   0.000     .2333266     .627469
           3 |  -.1379211   .1109862    -1.24   0.215    -.3564294    .0805873
           6 |  -.2449897   .1219734    -2.01   0.046    -.4851295   -.0048499
          12 |   .0609285   .1069997     0.57   0.570    -.1497314    .2715884
          15 |   .0320104   .1129134     0.28   0.777    -.1902922     .254313
          18 |  -.1373498   .1236824    -1.11   0.268    -.3808542    .1061547
          21 |   .1193651   .1219734     0.98   0.329    -.1207747    .3595049
          24 |   .7679883   .1139662     6.74   0.000     .5436129    .9923637
          27 |   .6948379   .1595527     4.35   0.000     .3807124    1.008963
             |
       _cons |   2.176791   .0778537    27.96   0.000     2.023513    2.330068
------------------------------------------------------------------------------
```

. xi:regress log10meannt ib12.ag13 // Base class --> 12-14 months of age

Source	SS	df	MS	Number of obs = 280
Model	26.678699	9	2.96429989	F (9, 270) = 15.28
Residual	52.3688042	270	.193958534	Prob > F = 0.0000
Total	79.0475032	279	.283324384	R-squared = 0.3375
				Adj R-squared = 0.3154
				Root MSE = .44041

log_{10}meannt	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
ag13						
0	.3694693	.0966751	3.82	0.000	.1791364	.5598021
3	-.1988496	.1079095	-1.84	0.066	-.4113007	.0136015
6	-.3059182	.1191807	-2.57	0.011	-.5405598	-.0712766
9	-.0609285	.1069997	-0.57	0.570	-.2715884	.1497314
15	-.0289181	.1098907	-0.26	0.793	-.2452697	.1874335
18	-.1982783	.1209291	-1.64	0.102	-.4363623	.0398057
21	.0584366	.1191807	0.49	0.624	-.1762051	.2930782
24	.7070598	.1109722	6.37	0.000	.4885789	.9255406
27	.6339094	.157428	4.03	0.000	.3239668	.943852
_cons	2.237719	.0734012	30.49	0.000	2.093208	2.382231

. xi:regress log_{10}meannt ib15.ag13 // Base class --> 15-17 months of age

Source	SS	df	MS	Number of obs = 280
Model	26.678699	9	2.96429989	F (9, 270) = 15.28
Residual	52.3688042	270	.193958534	Prob > F = 0.0000
Total	79.0475032	279	.283324384	R-squared = 0.3375
				Adj R-squared = 0.3154
				Root MSE = .44041

log_{10}meannt	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
ag13						
0	.3983873	.1031822	3.86	0.000	.1952434	.6015313
3	-.1699315	.1137759	-1.49	0.136	-.3939322	.0540692
6	-.2770001	.1245172	-2.22	0.027	-.5221481	-.0318521
9	-.0320104	.1129134	-0.28	0.777	-.254313	.1902922
12	.0289181	.1098907	0.26	0.793	-.1874335	.2452697
18	-.1693602	.1261917	-1.34	0.181	-.4178051	.0790846
21	.0873546	.1245172	0.70	0.484	-.1577934	.3325027
24	.7359779	.1166847	6.31	0.000	.5062503	.9657054
27	.6628275	.1615057	4.10	0.000	.3448569	.9807981
_cons	2.208801	.0817816	27.01	0.000	2.04779	2.369812

. xi:regress log_{10}meannt ib18.ag13 // Base class --> 18-20 months of age

Source	SS	df	MS	Number of obs =	280
Model	26.678699	9	2.96429989	F (9, 270) =	15.28
Residual	52.3688042	270	.193958534	Prob > F =	0.0000
Total	79.0475032	279	.283324384	R-squared =	0.3375
				Adj R-squared =	0.3154
				Root MSE =	.44041

log_{10}meannt	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
ag13						
0	.5677476	.1148671	4.94	0.000	.3415984	.7938967
3	-.0005713	.1244703	-0.00	0.996	-.245627	.2444844
6	-.1076399	.1343593	-0.80	0.424	-.372165	.1568852
9	.1373498	.1236824	1.11	0.268	-.1061547	.3808542
12	.1982783	.1209291	1.64	0.102	-.0398057	.4363623
15	.1693602	.1261917	1.34	0.181	-.0790846	.4178051
21	.2567149	.1343593	1.91	0.057	-.0078102	.5212399
24	.9053381	.1271346	7.12	0.000	.6550368	1.155639
27	.8321877	.1692099	4.92	0.000	.4990492	1.165326
_cons	2.039441	.0961047	21.22	0.000	1.850231	2.228651

. xi:regress log_{10}meannt ib21.ag13 // Base class --> 21-23 months of age

Source	SS	df	MS	Number of obs =	280
Model	26.678699	9	2.96429989	F (9, 270) =	15.28
Residual	52.3688042	270	.193958534	Prob > F =	0.0000
Total	79.0475032	279	.283324384	R-squared =	0.3375
				Adj R-squared =	0.3154
				Root MSE =	.44041

log_{10}meannt	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
ag13						
0	.3110327	.1130249	2.75	0.006	.0885105	.5335549
3	-.2572861	.1227722	-2.10	0.037	-.4989988	-.0155735
6	-.3643548	.1327878	-2.74	0.006	-.6257859	-.1029236
9	-.1193651	.1219734	-0.98	0.329	-.3595049	.1207747
12	-.0584366	.1191807	-0.49	0.624	-.2930782	.1762051
15	-.0873546	.1245172	-0.70	0.484	-.3325027	.1577934
18	-.2567149	.1343593	-1.91	0.057	-.5212399	.0078102
24	.6486232	.1254727	5.17	0.000	.401594	.8956524
27	.5754728	.1679647	3.43	0.001	.2447857	.90616

```
             |
       _cons |   2.296156   .0938951    24.45   0.000     2.111296    2.481015
------------------------------------------------------------------------------
```

. xi:regress log_{10}meannt ib24.ag13 // Base class --> 24-26 months of age

```
      Source |       SS       df       MS              Number of obs =     280
-------------+------------------------------           F(  9,   270) =   15.28
       Model |  26.678699      9  2.96429989           Prob > F      =  0.0000
    Residual |  52.3688042   270  .193958534           R-squared     =  0.3375
-------------+------------------------------           Adj R-squared =  0.3154
       Total |  79.0475032   279  .283324384           Root MSE      =  .44041

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       ag13 |
          0 |  -.3375905   .1043333    -3.24   0.001    -.5430007   -.1321804
          3 |  -.9059094   .1148208    -7.89   0.000    -1.131967   -.6798514
          6 |  -1.012978   .1254727    -8.07   0.000    -1.260007   -.7659488
          9 |  -.7679883   .1139662    -6.74   0.000    -.9923637   -.5436129
         12 |  -.7070598   .1109722    -6.37   0.000    -.9255406   -.4885789
         15 |  -.7359779   .1166847    -6.31   0.000    -.9657054   -.5062503
         18 |  -.9053381   .1271346    -7.12   0.000    -1.155639   -.6550368
         21 |  -.6486232   .1254727    -5.17   0.000    -.8956524    -.401594
         27 |  -.0731504   .1622435    -0.45   0.652    -.3925736    .2462728
            |
       _cons |   2.944779   .0832291    35.38   0.000     2.780918     3.10864
------------------------------------------------------------------------------
```

```
//=============================================================================
// Code: This code generates a binary variable based on a putative neutralising
//       antibody protective threshold obtained from Glezen et al.  (1981). On the
basis
//       of this variable, survival analysis is carried out to determine the mean
//       duration of protection by maternal antibodies and the age prevalence of these
//       protective titres
//=============================================================================
```

. use "C:\Documents and Settings\csande.KWTRP\Desktop\paper2\Full_COHORT_neut.dta",
clear

. gen prot_sev=.

. replace prot_sev=1 if log_{10}meannt>=2.48

```
. replace prot_sev=0 if log10meannt<2.48

. gen fail=1

. replace fail=0 if prot_sev==1
  (106 real changes made)

. replace fail=1 if sample_d>=inf1_d
  (80 real changes made)


. bysort sample: egen mx=max  (sample_d)

. format mx %d

. stset  sample_d, id  ( sample) f  (fail) or  (d_birth) ex  ( mx) sc  (30.5)

                id:  sample
     failure event:  fail != 0 & fail < .
obs. time interval:  (sample_d[_n-1], sample_d]
 exit on or before:  time mx
   t for analysis:  (time-origin)/30.5
            origin:  time d_birth

--------------------------------------------------------------------------------
      280  total obs.
       24  obs. end on or before enter  ()
--------------------------------------------------------------------------------
      256  obs. remaining, representing
       28  subjects
      251  failures in multiple failure-per-subject data
 696.1311  total analysis time at risk, at risk from t =         0
                              earliest observed entry t =         0
                                   last observed exit t =  29.93443

. strate

        failure _d:  fail
  analysis time _t:  (sample_d-origin)/30.5
            origin:  time d_birth
 exit on or before:  time mx
                id:  sample

Estimated rates and lower/upper bounds of 95% confidence intervals
  (256 records included in the analysis)

  +-----------------------------------------------+
  |   D          Y      Rate     Lower     Upper |
  |-----------------------------------------------|
  | 251   696.1311   0.36056   0.31861   0.40805 |
  +-----------------------------------------------+
```

```
. di 1/0.36056
2.7734635


//age prevalence of protective antibodies

. ci prot_sev if sample_d<= infl_d,by  (ag6) binomial  //calculate binomial
proportions

-------------------------------------------------------------------------------
-> ag6 = 0

                                                    -- Binomial Exact --
    Variable |        Obs        Mean    Std. Err.       [95% Conf. Interval]
-------------+-------------------------------------------------------------
    prot_sev |         24           1           0        .8575264           1*

  (*) one-sided, 97.5% confidence interval

-------------------------------------------------------------------------------
-------------------------------
-> ag6 = 1

                                                    -- Binomial Exact --
    Variable |        Obs        Mean    Std. Err.       [95% Conf. Interval]
-------------+-------------------------------------------------------------
    prot_sev |          0           .           .                .           .

-------------------------------------------------------------------------------
-------------------------------
-> ag6 = 2

                                                    -- Binomial Exact --
    Variable |        Obs        Mean    Std. Err.       [95% Conf. Interval]
-------------+-------------------------------------------------------------
    prot_sev |         19    .3684211    .1106647        .1628859    .6164221

-------------------------------------------------------------------------------
-------------------------------
-> ag6 = 3

                                                    -- Binomial Exact --
    Variable |        Obs        Mean    Std. Err.       [95% Conf. Interval]
-------------+-------------------------------------------------------------
    prot_sev |         16           0           0                0    .2059072*

  (*) one-sided, 97.5% confidence interval
```

```
-------------------------------------------------------------------------------
------------------------------
-> ag6 = 6

                                                    -- Binomial Exact --
    Variable |        Obs        Mean    Std. Err.       [95% Conf. Interval]
-------------+---------------------------------------------------------------
    prot_sev |         22           0           0                0    .1543725*

  (*) one-sided, 97.5% confidence interval

-------------------------------------------------------------------------------
------------------------------
-> ag6 = 12

                                                    -- Binomial Exact --
    Variable |        Obs        Mean    Std. Err.       [95% Conf. Interval]
-------------+---------------------------------------------------------------
    prot_sev |          9    .1111111    .1047566        .0028091    .4824965
```

```
//=============================================================================
// Code: This code calculates the duration of neutralising antibodies following
//       primary infection. It calculates the mean neutralising antibody titre at
//       different time points pre and post infection. A pre-infection control which
//       encompasses neutralising titres up to six months prior to primary
//       infection is defined.
//
//=============================================================================


. gen days_post_infl=sample_d-infl_d  //days pre/post RSV Positive sample for first
identified infection

. gen  months_post_infl= days_post_infl/30.5  // the number of months before or after
the date of the first identified infection

. //egen months_post_infl_classes=cut  (months_post_infl),at  (-6,0,1,2,3,4,5,6)
. egen months_post_infl_classes=cut  (months_post_infl),at  (-6,0,0.5,1,2,3,4,5,6)

. replace months_post_infl_classes=. if age_m<5 & months_post_infl_classes==-6 //get
rid maternal antibodies in pre exposure control
  (8 real changes made, 8 to missing)

. graph box log10meannt if sample_d>=infl_d-90 & sample_d<= date_resp1_end,over  (
months_post_infl_classes) // box plot
```

```
. xi:regress log10meannt i.months_post_infl_classes age_m,cluster  (sample) // means
compared using regression model with clustered sandwich estimation for repeated
measurements

i.months_post~s   _Imonths_po_1-8  (_Imonths_po_1 for months_~s==-6 omitted)

Linear regression                                      Number of obs =     123
                                                       F  (  8,    27) =   68.71
                                                       Prob > F      =  0.0000
                                                       R-squared     =  0.6553
                                                       Root MSE      =  .25337

                    (Std. Err. adjusted for 28 clusters in sample)
------------------------------------------------------------------------------
             |               Robust
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Imonths_p~2 |   .0977815   .0653437     1.50   0.146    -.0362927    .2318556
_Imonths_p~3 |   .9803547   .0586526    16.71   0.000     .8600095     1.1007
_Imonths_p~4 |   .7657186   .0627076    12.21   0.000     .6370532    .8943839
_Imonths_p~5 |   .4809447   .0799101     6.02   0.000     .3169828    .6449066
_Imonths_p~6 |    .214715   .1056726     2.03   0.052    -.0021073    .4315373
_Imonths_p~7 |   .2072252   .1112533     1.86   0.073    -.0210477    .4354981
_Imonths_p~8 |   .0963621   .0801376     1.20   0.240    -.0680667    .2607908
       age_m |   .0043244   .0061555     0.70   0.488    -.0083057    .0169544
       _cons |    1.77324   .0569952    31.11   0.000     1.656295    1.890184
------------------------------------------------------------------------------
```

```
//=============================================================================
// Code: This code compares the mean neutralising titres at the acute stage of primary
//       and secondary infection using a linear regression model
//
//=============================================================================
```

```
. gen epid=.  //generate epidemic start and end boundaries
  (280 missing values generated)

.
. replace epid=1 if sample_d >td  (01/03/2002) & sample_d <td  (01/08/2002) & epid==.
  (35 real changes made)

. replace epid=2 if sample_d >td  (01/12/2002) & sample_d <td  (01/05/2003) & epid==.
  (45 real changes made)

. replace epid=3 if sample_d >td  (01/12/2003) & sample_d <td  (01/07/2004) & epid==.
  (71 real changes made)
```

```
. replace epid=4 if sample_d >td  (01/11/2004) & sample_d <td  (01/04/2005) & epid==.
  (15 real changes made)

. replace epid=5 if sample_d >td  (01/11/2005) & sample_d <td  (01/08/2006) & epid==.
  (0 real changes made)

. replace epid=6 if sample_d >td  (01/12/2006) & sample_d <td  (01/05/2007) & epid==.
  (0 real changes made)

. replace epid=7 if sample_d >td  (01/11/2007) & sample_d <td  (01/06/2008) & epid==.
  (0 real changes made)

. replace epid=8 if sample_d >td  (01/11/2008) & sample_d <td  (01/06/2009) & epid==.
  (0 real changes made)

. replace epid=9 if sample_d >td  (01/11/2009) & sample_d <td  (01/07/2010) & epid==.
  (0 real changes made)



. replace epid=0 if epid==.   //define inter-epidemic period
  (114 real changes made)



gen displ_d1=sample_d-inf1_d   //displacement in days between sample date and positive
ifat sample date in primary infection
gen displ_d2=sample_d-inf2_d   //displacement in days between sample date and positive
ifat sample date in secondary infection

gen d1= displ_d1  if  displ_d1 >=0 &  displ_d1<=10  //pick only the samples collected
within 10 days of infection and assume these are "acute" samples
gen d2= displ_d2  if  displ_d2 >=0 &  displ_d2<=10

gen dis  // contains 1 or 2 if sample fulfills the "acute" sample criteria  (collected
<=10 days after IFAT pos sample)
        // for primary & secondary infection respectively

replace dis=1 if d1!=.
replace dis=2 if d2!=.


. gen dis2=.  //initially copy of dis
  (280 missing values generated)

. replace dis2=1 if dis==1
  (24 real changes made)

. replace dis2=2 if dis==2
  (9 real changes made)
```

```
. replace dis2=0 if months_post_inf1_classes==-6 //pre-infection control group
  (28 real changes made)

. xi:regress log10meannt i.dis2   //comparison of primary and secondary and intra epid
ab at first 10 days. Base class is the pre-infection control
i.dis2            _Idis2_0-2  (naturally coded; _Idis2_0 omitted)

      Source |       SS       df       MS              Number of obs =      61
-------------+------------------------------           F (  2,    58) =   62.45
       Model |  10.5272816     2  5.26364081           Prob > F      =  0.0000
    Residual |  4.88882252    58  .084290043           R-squared     =  0.6829
-------------+------------------------------           Adj R-squared =  0.6719
       Total |  15.4161041    60  .256935069           Root MSE      =  .29033

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Idis2_1 |    .099774   .0807616     1.24   0.222     -.061888     .261436
    _Idis2_2 |   1.210256   .1112471    10.88   0.000      .9875711    1.432942
       _cons |   1.815324   .0548667    33.09   0.000     1.705496    1.925152
------------------------------------------------------------------------------

xi:regress log10meannt ib1.dis2  //base class -> primary infection acute samples

      Source |       SS       df       MS              Number of obs =      61
-------------+------------------------------           F (  2,    58) =   62.45
       Model |  10.5272816     2  5.26364081           Prob > F      =  0.0000
    Residual |  4.88882252    58  .084290043           R-squared     =  0.6829
-------------+------------------------------           Adj R-squared =  0.6719
       Total |  15.4161041    60  .256935069           Root MSE      =  .29033

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
        dis2 |
          0  |   -.099774   .0807616    -1.24   0.222     -.261436     .061888
          2  |   1.110482   .1134797     9.79   0.000      .883328    1.337637
             |
       _cons |   1.915098   .0592628    32.32   0.000      1.79647    2.033725
------------------------------------------------------------------------------


. ciplot log10meannt,by  (dis2)


//=============================================================================
// Code: This code generates summary plots of the mean PRNTs in 3 calendar month
```

```
//       strata for cohort 1 and 2 and compares the mean PRNTs at one time point to
//       the next time point in the series using a regression model. The plots are
//       generated relative to temporal population level transmission estimates
//       obtained from inpatient surveillance data
//
//===============================================================================

.
. // use this module to plot compare the kinetics of the neut response in the 2
cohorts to seasonal rsv transmission
. use "C:\Documents and
Settings\csande.KWTRP\Desktop\paper2\Full_COHORT_neut_surveillance overlay.dta",clear
// dataset contains a merge of kinetics data and inpatient surveillance data


. summ sample_d,d  // get the range of sample dates for all infant samples in order to
generate appropriate calender strata

                        sample_d
-------------------------------------------------------------
      Percentiles      Smallest
 1%         15387          15372
 5%         15455          15381
10%         15491          15387       Obs                 280
25%         15667          15389       Sum of Wgt.         280

50%       15906.5                      Mean           15908.95
                        Largest       Std. Dev.       294.4396
75%       16162.5          16483
90%       16273.5          16490       Variance        86694.7
95%         16382          16493       Skewness       .0204077
99%         16490          16572       Kurtosis       1.989988


. egen sample_d_class=cut  (sample_d),at  ( 15372  (90)16572) // generate 3 calender
month strata of all samples in both cohort 1 and 2
  (2047 missing values generated)

. collapse  (mean) l10=log10meannt  (sd) sd10=log10meannt  (count) cnt = log10meannt,by
(sample_d_class cohort) // calculate mean, SDs and Ns for each stratum

. generate hi =  l10+ invttail  (cnt-1,0.025)*  (sd10 / sqrt  (cnt)) // calculate
upper confidence limit

. generate lo =  l10- invttail  (cnt-1,0.025)*  (sd10 / sqrt  (cnt)) // calculate
lower confidence limit


. capture: rename cohort cohort2
```

```
. append using "C:\Documents and
Settings\csande.KWTRP\Desktop\paper2\Full_COHORT_neut_surveillance overlay.dta"
//append to kinetics/IP dataset
  (label ab already defined)


// generate log normalised confidence estimates for cohort 1 and 2

. capture: gen l10a=l10 if cohort2==1

. capture: gen l10b=l10 if cohort2==2

. capture: gen hia=hi if cohort2==1

. capture: gen hib=hi if cohort2==2

. capture: gen loa=lo if cohort2==1

. capture: gen lob=lo if cohort2==2


. egen sc=cut  (sample_d),at  ( 15372  (90)16572) // re-generate 3 monthly strata

.
. //neut kinetics/tranmission plots
. // cohort 1
. twoway  (scatter  l10a  sample_d_class, connect  (direct) yaxis  (1))  (rcap hia loa
sample_d_cla
> ss, yaxis  (1))  (bar weekly_adm_tot weekly_adm if d_admin<=td  (01jan2006) &
serial_n==1,yaxi
> s  (2))


. xi:regress log10meannt ib15372.sc if cohort==1   // compare the mean titre of stratum
1 to that of stratum 2 for cohort 1

      Source |       SS       df       MS              Number of obs =     171
-------------+------------------------------           F  ( 10,   160) =   11.56
       Model |  21.5204857    10  2.15204857           Prob > F      =  0.0000
    Residual |  29.7801855   160   .18612616           R-squared     =  0.4195
-------------+------------------------------           Adj R-squared =  0.3832
       Total |  51.3006712   170  .301768654           Root MSE      =  .43142

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
       15462  |  -.5725675   .1450861    -3.95   0.000    -.8590983   -.2860367
       15552  |  -.9220406   .1557027    -5.92   0.000    -1.229538   -.6145431
       15642  |  -.9755165    .166169    -5.87   0.000    -1.303684   -.6473491
```

```
      15732 |  -.5035725   .1462435    -3.44   0.001    -.7923891    -.214756
      15822 |  -.5084333   .1630627    -3.12   0.002    -.8304661   -.1864005
      15912 |   -.810761    .160322    -5.06   0.000    -1.127381   -.4941409
      16002 |  -.8729008   .1843242    -4.74   0.000    -1.236923   -.5088787
      16092 |  -.1792767    .153737    -1.17   0.245    -.4828922    .1243387
      16182 |   .0822558   .1519567     0.54   0.589    -.2178438    .3823553
      16272 |  -.0582289   .2247665    -0.26   0.796    -.5021205    .3856627
            |
      _cons |   2.840375   .1153028    24.63   0.000     2.612663    3.068086
------------------------------------------------------------------------------
```

. xi:regress log_{10}meannt ib15462.sc if cohort==1 // compare the mean titre of stratum 2 to that of stratum 3 for cohort 1

```
      Source |       SS       df       MS              Number of obs =     171
-------------+------------------------------           F  ( 10,   160) =   11.56
       Model |  21.5204857    10  2.15204857           Prob > F      =  0.0000
    Residual |  29.7801855   160   .18612616           R-squared     =  0.4195
-------------+------------------------------           Adj R-squared =  0.3832
       Total |  51.3006712   170  .301768654           Root MSE      =  .43142

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
      15372 |   .5725675   .1450861     3.95   0.000     .2860367    .8590983
      15552 |  -.3494731    .136762    -2.56   0.012    -.6195647   -.0793816
      15642 |   -.402949   .1485687    -2.71   0.007    -.6963575   -.1095405
      15732 |   .0689949   .1258876     0.55   0.584    -.1796208    .3176106
      15822 |   .0641342   .1450861     0.44   0.659    -.2223966     .350665
      15912 |  -.2381936   .1419988    -1.68   0.095    -.5186273    .0422402
      16002 |  -.3003333   .1686296    -1.78   0.077    -.6333602    .0326935
      16092 |   .3932907   .1345199     2.92   0.004     .1276272    .6589542
      16182 |   .6548232   .1324816     4.94   0.000     .3931851    .9164613
      16272 |   .5143386    .212086     2.43   0.016     .0954895    .9331876
            |
      _cons |   2.267807   .0880639    25.75   0.000      2.09389    2.441725
------------------------------------------------------------------------------
```

. xi:regress log_{10}meannt ib15552.sc if cohort==1 // compare the mean titre of stratum 3 to that of stratum 4 for cohort 1

```
      Source |       SS       df       MS              Number of obs =     171
-------------+------------------------------           F  ( 10,   160) =   11.56
       Model |  21.5204857    10  2.15204857           Prob > F      =  0.0000
    Residual |  29.7801855   160   .18612616           R-squared     =  0.4195
-------------+------------------------------           Adj R-squared =  0.3832
       Total |  51.3006712   170  .301768654           Root MSE      =  .43142

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
```

sc						
15372	.9220406	.1557027	5.92	0.000	.6145431	1.229538
15462	.3494731	.136762	2.56	0.012	.0793816	.6195647
15642	-.0534759	.1589528	-0.34	0.737	-.367392	.2604403
15732	.4184681	.1379893	3.03	0.003	.1459528	.6909833
15822	.4136073	.1557027	2.66	0.009	.1061098	.7211048
15912	.1112796	.15283	0.73	0.468	-.1905446	.4131038
16002	.0491398	.1778462	0.28	0.783	-.302089	.4003686
16092	.7427639	.1459073	5.09	0.000	.4546113	1.030916
16182	1.004296	.1440302	6.97	0.000	.7198508	1.288742
16272	.8638117	.2194854	3.94	0.000	.4303497	1.297274
_cons	1.918334	.1046355	18.33	0.000	1.711689	2.124979

. xi:regress $\log_{10}$meannt ib15642.sc if cohort==1 // compare the mean titre of stratum 4 to that of stratum 5 for cohort 1

Source	SS	df	MS	Number of obs =	171
Model	21.5204857	10	2.15204857	F (10, 160) =	11.56
Residual	29.7801855	160	.18612616	Prob > F =	0.0000
Total	51.3006712	170	.301768654	R-squared =	0.4195
				Adj R-squared =	0.3832
				Root MSE =	.43142

$\log_{10}$meannt	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
sc						
15372	.9755165	.166169	5.87	0.000	.6473491	1.303684
15462	.402949	.1485687	2.71	0.007	.1095405	.6963575
15552	.0534759	.1589528	0.34	0.737	-.2604403	.367392
15732	.471944	.1496992	3.15	0.002	.1763028	.7675851
15822	.4670832	.166169	2.81	0.006	.1389158	.7952505
15912	.1647555	.1634803	1.01	0.315	-.158102	.487613
16002	.1026157	.1870777	0.55	0.584	-.2668444	.4720758
16092	.7962397	.1570278	5.07	0.000	.4861252	1.106354
16182	1.057772	.1552853	6.81	0.000	.7510992	1.364445
16272	.9172876	.22703	4.04	0.000	.4689256	1.36565
_cons	1.864858	.1196553	15.59	0.000	1.628551	2.101166

. xi:regress $\log_{10}$meannt ib15732.sc if cohort==1 // compare the mean titre of stratum 5 to that of stratum 6 for cohort 1

Source	SS	df	MS	Number of obs =	171
Model	21.5204857	10	2.15204857	F (10, 160) =	11.56
Residual	29.7801855	160	.18612616	Prob > F =	0.0000
				R-squared =	0.4195

```
-------------+------------------------------           Adj R-squared =  0.3832
       Total |  51.3006712   170  .301768654           Root MSE      =  .43142

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
       15372 |   .5035725   .1462435     3.44   0.001      .214756    .7923891
       15462 |  -.0689949   .1258876    -0.55   0.584    -.3176106    .1796208
       15552 |  -.4184681   .1379893    -3.03   0.003    -.6909833   -.1459528
       15642 |   -.471944   .1496992    -3.15   0.002    -.7675851   -.1763028
       15822 |  -.0048608   .1462435    -0.03   0.974    -.2936773    .2839558
       15912 |  -.3071885   .1431812    -2.15   0.033    -.5899572   -.0244197
       16002 |  -.3693283   .1696264    -2.18   0.031    -.7043238   -.0343328
       16092 |   .3242958   .1357674     2.39   0.018     .0561685     .592423
       16182 |   .5858283   .1337481     4.38   0.000      .321689    .8499676
       16272 |   .4453436   .2128795     2.09   0.038     .0249276    .8657596
             |
       _cons |   2.336802    .089958    25.98   0.000     2.159144     2.51446
------------------------------------------------------------------------------
```

. xi:regress log10meannt ib15822.sc if cohort==1 // compare the mean titre of stratum 6 to that of stratum 7 for cohort 1

```
      Source |       SS       df       MS              Number of obs =     171
-------------+------------------------------           F  ( 10,   160) =   11.56
       Model |  21.5204857    10  2.15204857           Prob > F      =  0.0000
    Residual |  29.7801855   160   .18612616           R-squared     =  0.4195
-------------+------------------------------           Adj R-squared =  0.3832
       Total |  51.3006712   170  .301768654           Root MSE      =  .43142

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
       15372 |   .5084333   .1630627     3.12   0.002     .1864005    .8304661
       15462 |  -.0641342   .1450861    -0.44   0.659     -.350665    .2223966
       15552 |  -.4136073   .1557027    -2.66   0.009    -.7211048   -.1061098
       15642 |  -.4670832    .166169    -2.81   0.006    -.7952505   -.1389158
       15732 |   .0048608   .1462435     0.03   0.974    -.2839558    .2936773
       15912 |  -.3023277    .160322    -1.89   0.061    -.6189478    .0142924
       16002 |  -.3644675   .1843242    -1.98   0.050    -.7284896   -.0004454
       16092 |   .3291566    .153737     2.14   0.034     .0255411     .632772
       16182 |   .5906891   .1519567     3.89   0.000     .2905895    .8907886
       16272 |   .4502044   .2247665     2.00   0.047     .0063128     .894096
             |
       _cons |   2.331941   .1153028    20.22   0.000      2.10423    2.559653
------------------------------------------------------------------------------
```

. xi:regress log10meannt ib15912.sc if cohort==1 // compare the mean titre of stratum 7 to that of stratum 8 for cohort 1

```
      Source |       SS       df       MS              Number of obs =     171
-------------+------------------------------           F  ( 10,   160) =   11.56
       Model |  21.5204857    10  2.15204857           Prob > F      =  0.0000
    Residual |  29.7801855   160   .18612616           R-squared     =  0.4195
-------------+------------------------------           Adj R-squared =  0.3832
       Total |  51.3006712   170  .301768654           Root MSE      =  .43142

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
       15372 |    .810761    .160322     5.06   0.000     .4941409    1.127381
       15462 |   .2381936   .1419988     1.68   0.095    -.0422402    .5186273
       15552 |  -.1112796     .15283    -0.73   0.468    -.4131038    .1905446
       15642 |  -.1647555   .1634803    -1.01   0.315     -.487613     .158102
       15732 |   .3071885   .1431812     2.15   0.033     .0244197    .5899572
       15822 |   .3023277    .160322     1.89   0.061    -.0142924    .6189478
       16002 |  -.0621398   .1819041    -0.34   0.733    -.4213824    .2971029
       16092 |   .6314843   .1508269     4.19   0.000      .333616    .9293525
       16182 |   .8930168   .1490118     5.99   0.000     .5987331      1.1873
       16272 |   .7525321   .2227861     3.38   0.001     .3125515    1.192513
             |
       _cons |   2.029614    .111393    18.22   0.000     1.809623    2.249604
------------------------------------------------------------------------------
```

. xi:regress log10meannt ib16002.sc if cohort==1 // compare the mean titre of stratum 8 to that of stratum 9 for cohort 1

```
      Source |       SS       df       MS              Number of obs =     171
-------------+------------------------------           F  ( 10,   160) =   11.56
       Model |  21.5204857    10  2.15204857           Prob > F      =  0.0000
    Residual |  29.7801855   160   .18612616           R-squared     =  0.4195
-------------+------------------------------           Adj R-squared =  0.3832
       Total |  51.3006712   170  .301768654           Root MSE      =  .43142

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
       15372 |   .8729008   .1843242     4.74   0.000     .5088787    1.236923
       15462 |   .3003333   .1686296     1.78   0.077    -.0326935    .6333602
       15552 |  -.0491398   .1778462    -0.28   0.783    -.4003686     .302089
       15642 |  -.1026157   .1870777    -0.55   0.584    -.4720758    .2668444
       15732 |   .3693283   .1696264     2.18   0.031     .0343328    .7043238
       15822 |   .3644675   .1843242     1.98   0.050     .0004454    .7284896
       15912 |   .0621398   .1819041     0.34   0.733    -.2971029    .4213824
       16092 |   .6936241   .1761279     3.94   0.000     .3457888    1.041459
       16182 |   .9551566   .1745761     5.47   0.000     .6103861    1.299927
       16272 |   .8146719   .2406365     3.39   0.001     .3394385    1.289905
             |
```

```
       _cons |   1.967474   .1438078    13.68   0.000     1.683468     2.25148
------------------------------------------------------------------------------
```

. xi:regress log_{10}meannt ib16092.sc if cohort==1 // compare the mean titre of stratum 9 to that of stratum 10 for cohort 1

```
      Source |       SS       df       MS              Number of obs =     171
-------------+------------------------------           F  ( 10,   160) =   11.56
       Model |  21.5204857    10  2.15204857           Prob > F      =  0.0000
    Residual |  29.7801855   160   .18612616           R-squared     =  0.4195
-------------+------------------------------           Adj R-squared =  0.3832
       Total |  51.3006712   170  .301768654           Root MSE      =  .43142

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
       15372 |   .1792767    .153737     1.17   0.245    -.1243387    .4828922
       15462 |  -.3932907   .1345199    -2.92   0.004    -.6589542   -.1276272
       15552 |  -.7427639   .1459073    -5.09   0.000    -1.030916   -.4546113
       15642 |  -.7962397   .1570278    -5.07   0.000    -1.106354   -.4861252
       15732 |  -.3242958   .1357674    -2.39   0.018     -.592423   -.0561685
       15822 |  -.3291566    .153737    -2.14   0.034     -.632772   -.0255411
       15912 |  -.6314843   .1508269    -4.19   0.000    -.9293525    -.333616
       16002 |  -.6936241   .1761279    -3.94   0.000    -1.041459   -.3457888
       16182 |   .2615325    .141903     1.84   0.067    -.0187119    .5417769
       16272 |   .1210478   .2180953     0.56   0.580     -.309669    .5517647
             |
       _cons |   2.661098   .1016875    26.17   0.000     2.460275    2.861921
------------------------------------------------------------------------------
```

. xi:regress log_{10}meannt ib16182.sc if cohort==1 // compare the mean titre of stratum 10 to that of stratum 11 for cohort 1

```
      Source |       SS       df       MS              Number of obs =     171
-------------+------------------------------           F  ( 10,   160) =   11.56
       Model |  21.5204857    10  2.15204857           Prob > F      =  0.0000
    Residual |  29.7801855   160   .18612616           R-squared     =  0.4195
-------------+------------------------------           Adj R-squared =  0.3832
       Total |  51.3006712   170  .301768654           Root MSE      =  .43142

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
       15372 |  -.0822558   .1519567    -0.54   0.589    -.3823553    .2178438
       15462 |  -.6548232   .1324816    -4.94   0.000    -.9164613   -.3931851
       15552 |  -1.004296   .1440302    -6.97   0.000    -1.288742   -.7198508
       15642 |  -1.057772   .1552853    -6.81   0.000    -1.364445   -.7510992
       15732 |  -.5858283   .1337481    -4.38   0.000    -.8499676    -.321689
       15822 |  -.5906891   .1519567    -3.89   0.000    -.8907886   -.2905895
```

```
      15912 |  -.8930168   .1490118    -5.99   0.000      -1.1873   -.5987331
      16002 |  -.9551566   .1745761    -5.47   0.000    -1.299927   -.6103861
      16092 |  -.2615325    .141903    -1.84   0.067    -.5417769    .0187119
      16272 |  -.1404847   .2168441    -0.65   0.518    -.5687303     .287761
            |
       _cons |    2.92263   .0989753    29.53   0.000     2.727164    3.118097
------------------------------------------------------------------------------

.
. //cohort 2
. twoway (scatter l10b sample_d_class, connect (direct) yaxis (1)) (rcap hib lob
sample_d_cl
> ass, yaxis (1)) (bar weekly_adm_tot weekly_adm if d_admin<=td (01jan2006) &
serial_n==1,ya
> xis (2))

. xi:regress log10meannt ib15642.sc if cohort==2 //compare the mean titre of stratum 1
to that of stratum 2 for cohort 2 - note stratum 1 for cohort 2
                                                //is stratum 2 for cohort 1 since
cohort 2 does not have data for the very first stratum as it had not
                                              // been recruited yet
      Source |       SS       df       MS              Number of obs =      83
-------------+------------------------------           F (  9,    73) =   11.50
       Model |  12.6901049     9  1.41001165           Prob > F      =  0.0000
    Residual |  8.95164222    73  .122625236           R-squared     =  0.5864
-------------+------------------------------           Adj R-squared =  0.5354
       Total |  21.6417471    82  .263923745           Root MSE      =  .35018

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
      15732 |  -.4948644   .2120438    -2.33   0.022    -.9174672   -.0722617
      15822 |  -.6964777   .1996327    -3.49   0.001    -1.094345   -.2986104
      15912 |  -1.109311   .2050437    -5.41   0.000    -1.517963   -.7006598
      16002 |  -1.139115   .2120438    -5.37   0.000    -1.561718   -.7165125
      16092 |  -1.013428    .182439    -5.55   0.000    -1.377028   -.6498272
      16182 |  -.4115317   .1808316    -2.28   0.026    -.7719286   -.0511349
      16272 |  -1.036245   .2214726    -4.68   0.000    -1.477639   -.5948511
      16362 |  -.7302438   .1918009    -3.81   0.000    -1.112502   -.3479852
      16452 |   .1069916   .2050437     0.52   0.603      -.30166    .5156432
            |
       _cons |   2.945961   .1566047    18.81   0.000     2.633848    3.258074
------------------------------------------------------------------------------
```

. xi:regress log_{10}meannt ib15732.sc if cohort==2 //compare the mean titre of stratum 2 to that of stratum 3 for cohort 2

Source	SS	df	MS	Number of obs =	83
Model	12.6901049	9	1.41001165	F (9, 73) =	11.50
Residual	8.95164222	73	.122625236	Prob > F =	0.0000
				R-squared =	0.5864
Total	21.6417471	82	.263923745	Adj R-squared =	0.5354
				Root MSE =	.35018

log_{10}meannt	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
sc						
15642	.4948644	.2120438	2.33	0.022	.0722617	.9174672
15822	-.2016133	.1891182	-1.07	0.290	-.5785253	.1752987
15912	-.614447	.1948215	-3.15	0.002	-1.002726	-.2261682
16002	-.6442508	.2021759	-3.19	0.002	-1.047187	-.2413149
16092	-.5185631	.1708698	-3.03	0.003	-.8591061	-.1780201
16182	.0833327	.1691525	0.49	0.624	-.2537877	.4204531
16272	-.5413808	.2120438	-2.55	0.013	-.9639835	-.1187781
16362	-.2353794	.1808316	-1.30	0.197	-.5957762	.1250175
16452	.601856	.1948215	3.09	0.003	.2135773	.9901348
_cons	2.451096	.1429599	17.15	0.000	2.166178	2.736015

. xi:regress log_{10}meannt ib15822.sc if cohort==2 //compare the mean titre of stratum 3 to that of stratum 4 for cohort 2

Source	SS	df	MS	Number of obs =	83
Model	12.6901049	9	1.41001165	F (9, 73) =	11.50
Residual	8.95164222	73	.122625236	Prob > F =	0.0000
				R-squared =	0.5864
Total	21.6417471	82	.263923745	Adj R-squared =	0.5354
				Root MSE =	.35018

log_{10}meannt	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
sc						
15642	.6964777	.1996327	3.49	0.001	.2986104	1.094345
15732	.2016133	.1891182	1.07	0.290	-.1752987	.5785253
15912	-.4128337	.1812348	-2.28	0.026	-.7740341	-.0516333
16002	-.4426375	.1891182	-2.34	0.022	-.8195495	-.0657255
16092	-.3169498	.1552002	-2.04	0.045	-.6262634	-.0076363
16182	.284946	.1533074	1.86	0.067	-.0205953	.5904873
16272	-.3397675	.1996327	-1.70	0.093	-.7376348	.0580998
16362	-.0337661	.1661044	-0.20	0.839	-.3648117	.2972796
16452	.8034693	.1812348	4.43	0.000	.4422689	1.16467

```
       _cons |   2.249483   .1238069    18.17   0.000     2.002736     2.49623
------------------------------------------------------------------------------
```

. xi:regress log_{10}meannt ib15912.sc if cohort==2 //compare the mean titre of stratum 4 to that of stratum 5 for cohort 2

```
      Source |       SS       df       MS              Number of obs =      83
-------------+------------------------------           F (  9,    73) =   11.50
       Model |  12.6901049     9  1.41001165           Prob > F      =  0.0000
    Residual |  8.95164222    73  .122625236           R-squared     =  0.5864
-------------+------------------------------           Adj R-squared =  0.5354
       Total |  21.6417471    82  .263923745           Root MSE      =  .35018

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
       15642 |   1.109311   .2050437     5.41   0.000     .7006598    1.517963
       15732 |    .614447   .1948215     3.15   0.002     .2261682    1.002726
       15822 |   .4128337   .1812348     2.28   0.026     .0516333    .7740341
       16002 |  -.0298038   .1948215    -0.15   0.879    -.4180826     .358475
       16092 |   .0958839   .1621013     0.59   0.556    -.2271836    .4189513
       16182 |   .6977797   .1602901     4.35   0.000     .3783221    1.017237
       16272 |   .0730662   .2050437     0.36   0.723    -.3355854    .4817178
       16362 |   .3790676    .17257     2.20   0.031     .0351361    .7229992
       16452 |   1.216303   .1871785     6.50   0.000     .8432569    1.589349
             |
       _cons |   1.836649   .1323552    13.88   0.000     1.572866    2.100433
------------------------------------------------------------------------------
```

. xi:regress log_{10}meannt ib16002.sc if cohort==2 //compare the mean titre of stratum 5 to that of stratum 6 for cohort 2

```
      Source |       SS       df       MS              Number of obs =      83
-------------+------------------------------           F (  9,    73) =   11.50
       Model |  12.6901049     9  1.41001165           Prob > F      =  0.0000
    Residual |  8.95164222    73  .122625236           R-squared     =  0.5864
-------------+------------------------------           Adj R-squared =  0.5354
       Total |  21.6417471    82  .263923745           Root MSE      =  .35018

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
       15642 |   1.139115   .2120438     5.37   0.000     .7165125    1.561718
       15732 |   .6442508   .2021759     3.19   0.002     .2413149    1.047187
       15822 |   .4426375   .1891182     2.34   0.022     .0657255    .8195495
       15912 |   .0298038   .1948215     0.15   0.879     -.358475    .4180826
       16092 |   .1256877   .1708698     0.74   0.464    -.2148553    .4662307
       16182 |   .7275835   .1691525     4.30   0.000     .3904631    1.064704
       16272 |    .10287    .2120438     0.49   0.629    -.3197328    .5254727
```

```
      16362 |   .4088714   .1808316     2.26   0.027     .0484746    .7692683
      16452 |   1.246107   .1948215     6.40   0.000     .8578281    1.634386
            |
      _cons |   1.806846   .1429599    12.64   0.000     1.521927    2.091764
------------------------------------------------------------------------------
```

. xi:regress $\log_{10}$meannt ib16092.sc if cohort==2 //compare the mean titre of stratum 6 to that of stratum 7 for cohort 2

```
      Source |       SS       df       MS              Number of obs =      83
-------------+------------------------------           F(  9,    73) =   11.50
       Model |  12.6901049     9  1.41001165           Prob > F      =  0.0000
    Residual |  8.95164222    73  .122625236           R-squared     =  0.5864
-------------+------------------------------           Adj R-squared =  0.5354
       Total |  21.6417471    82  .263923745           Root MSE      =  .35018

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
      15642  |   1.013428    .182439     5.55   0.000     .6498272    1.377028
      15732  |   .5185631   .1708698     3.03   0.003     .1780201    .8591061
      15822  |   .3169498   .1552002     2.04   0.045     .0076363    .6262634
      15912  |  -.0958839   .1621013    -0.59   0.556    -.4189513    .2271836
      16002  |  -.1256877   .1708698    -0.74   0.464    -.4662307    .2148553
      16182  |   .6018958   .1301306     4.63   0.000      .342546    .8612457
      16272  |  -.0228177    .182439    -0.13   0.901    -.3864181    .3407828
      16362  |   .2831838   .1449878     1.95   0.055    -.0057766    .5721441
      16452  |   1.120419   .1621013     6.91   0.000     .7973517    1.443487
             |
      _cons  |   1.932533   .0935892    20.65   0.000      1.74601    2.119056
------------------------------------------------------------------------------
```

. xi:regress $\log_{10}$meannt ib16182.sc if cohort==2 //compare the mean titre of stratum 7 to that of stratum 8 for cohort 2

```
      Source |       SS       df       MS              Number of obs =      83
-------------+------------------------------           F(  9,    73) =   11.50
       Model |  12.6901049     9  1.41001165           Prob > F      =  0.0000
    Residual |  8.95164222    73  .122625236           R-squared     =  0.5864
-------------+------------------------------           Adj R-squared =  0.5354
       Total |  21.6417471    82  .263923745           Root MSE      =  .35018

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
      15642  |   .4115317   .1808316     2.28   0.026     .0511349    .7719286
      15732  |  -.0833327   .1691525    -0.49   0.624    -.4204531    .2537877
      15822  |   -.284946   .1533074    -1.86   0.067    -.5904873    .0205953
      15912  |  -.6977797   .1602901    -4.35   0.000    -1.017237   -.3783221
```

```
      16002 |  -.7275835   .1691525    -4.30   0.000    -1.064704   -.3904631
      16092 |  -.6018958   .1301306    -4.63   0.000    -.8612457    -.342546
      16272 |  -.6247135   .1808316    -3.45   0.001    -.9851103   -.2643167
      16362 |  -.3187121   .1429599    -2.23   0.029    -.6036308   -.0337934
      16452 |   .5185233   .1602901     3.23   0.002     .1990657    .837981
            |
      _cons |   2.534429   .0904158    28.03   0.000     2.354231    2.714628
------------------------------------------------------------------------------
```

. xi:regress log_{10}meannt ib16272.sc if cohort==2 //compare the mean titre of stratum 8 to that of stratum 9 for cohort 2

```
      Source |       SS       df       MS              Number of obs =      83
-------------+------------------------------           F (  9,    73) =   11.50
       Model |  12.6901049     9  1.41001165           Prob > F      =  0.0000
    Residual |  8.95164222    73  .122625236           R-squared     =  0.5864
-------------+------------------------------           Adj R-squared =  0.5354
       Total |  21.6417471    82  .263923745           Root MSE      =  .35018

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
      15642  |   1.036245   .2214726     4.68   0.000     .5948511    1.477639
      15732  |   .5413808   .2120438     2.55   0.013     .1187781    .9639835
      15822  |   .3397675   .1996327     1.70   0.093    -.0580998    .7376348
      15912  |  -.0730662   .2050437    -0.36   0.723    -.4817178    .3355854
      16002  |    -.10287   .2120438    -0.49   0.629    -.5254727    .3197328
      16092  |   .0228177    .182439     0.13   0.901    -.3407828    .3864181
      16182  |   .6247135   .1808316     3.45   0.001     .2643167    .9851103
      16362  |   .3060014   .1918009     1.60   0.115    -.0762571       .68826
      16452  |   1.143237   .2050437     5.58   0.000     .7345852    1.551888
             |
      _cons  |   1.909716   .1566047    12.19   0.000     1.597603    2.221828
------------------------------------------------------------------------------
```

. xi:regress log_{10}meannt ib16362.sc if cohort==2 //compare the mean titre of stratum 9 to that of stratum 10 for cohort 2

```
      Source |       SS       df       MS              Number of obs =      83
-------------+------------------------------           F (  9,    73) =   11.50
       Model |  12.6901049     9  1.41001165           Prob > F      =  0.0000
    Residual |  8.95164222    73  .122625236           R-squared     =  0.5864
-------------+------------------------------           Adj R-squared =  0.5354
       Total |  21.6417471    82  .263923745           Root MSE      =  .35018

------------------------------------------------------------------------------
 log10meannt |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          sc |
      15642  |   .7302438   .1918009     3.81   0.000     .3479852    1.112502
```

```
       15732 |   .2353794   .1808316     1.30   0.197    -.1250175    .5957762
       15822 |   .0337661   .1661044     0.20   0.839    -.2972796    .3648117
       15912 |  -.3790676     .17257    -2.20   0.031    -.7229992   -.0351361
       16002 |  -.4088714   .1808316    -2.26   0.027    -.7692683   -.0484746
       16092 |  -.2831838   .1449878    -1.95   0.055    -.5721441    .0057766
       16182 |   .3187121   .1429599     2.23   0.029     .0337934    .6036308
       16272 |  -.3060014   .1918009    -1.60   0.115      -.68826    .0762571
       16452 |   .8372354     .17257     4.85   0.000     .4933038    1.181167
             |
       _cons |   2.215717   .1107363    20.01   0.000      1.99502    2.436414
------------------------------------------------------------------------------
```

APPENDIX 3

Matlab Code: Multi Dimensional Scaling of RSV B genetic relationships

Pairwise distances generated using MEGA software

```
%
=========================================================================
====
% This code performs multi dimensional scaling analysis using
distance data
% obtained from MEGA using RSV B G gene sequences obtained from
infants
% infected with different RSV B strains. Also different test strains
are
% included for comparison
%=========================================================================
======

clear
clc
% change working directory
chdir 'C:\Documents and Settings\csande.KWTRP\Desktop\paper1\thesis
chapter 7\'

%load data
RSVB_data = textread  ('C:\Documents and
Settings\csande.KWTRP\Desktop\paper1\thesis chapter 7\distance matrix
- RSV Bs-matlab-pded.csv', '', 'delimiter', ',', 'emptyvalue', NaN)

%run classical mds
[ya,e]=cmdscale  (RSVB_data)

%truncate the mds matrix to strain components
ken_ba_2008=ya  (1:1,:)
BA=ya  (2:21,:)
swe_b_1960=ya  (22:22,:)
SAB=ya  (23:42,:)
%A2=ya  (41:41,:)
```

```
%plot the data - 2d



%SAB infant samples
plot  (SAB  (:,1),SAB  (:,2),'bo',
'MarkerEdgeColor','b','Markersize',12,'MarkerFaceColor','y')
%text  (SAB  (:,1),SAB  (:,2),'SAB')
hold on



%ba infant samples
plot  (BA  (:,1),BA  (:,2),'bo',
'MarkerEdgeColor','b','Markersize',12,'MarkerFaceColor','g')
%text  (BA  (:,1),BA  (:,2),'BA')
hold on

%swe_b test strain
plot  (swe_b_1960  (:,1),swe_b_1960  (:,2),'bo',
'MarkerEdgeColor','b','Markersize',12,'MarkerFaceColor','b')
%text  (swe_b_1960  (:,1),swe_b_1960  (:,2),'Swe/B/1960')
hold on

%ken_ba test strain
plot  (ken_ba_2008  (:,1),ken_ba_2008  (:,2),'bo',
'MarkerEdgeColor','b','Markersize',12,'MarkerFaceColor','r')
%text  (ken_ba_2008  (:,1),ken_ba_2008  (:,2),'Ken/BA/2008')
hold on

legend  ('Contemporary non-BA strains  (SAB)','Contemporary BA
strains','Swe/B/1960','Ken/B/2008')
 grid on


%
==========================================================================
====
% This code performs multi dimensional scaling analysis using cross
```

```
% reactive neutralising antibody data against representative BA and
non-BA
% strains of RSV B. Test sera was obtained from infants who underwent
% natural infection with BA and non-BA strains. Reactivity is
expressed in
% terms of fold rise in titre from the acute to convalescent phases
of
% infection
%=====================================================================
======


% change working directory
chdir 'C:\Documents and Settings\csande.KWTRP\Desktop\paper1\thesis
chapter 7\'

%load data
RSVB_data = textread  ('C:\Documents and
Settings\csande.KWTRP\Desktop\paper1\thesis chapter 7\RSV_B_mds_data-
matlab matrix.csv', '', 'delimiter', ',', 'emptyvalue', NaN)

%generate p distances and reformat the matrix to square form
a=pdist  (RSVB_data)
b=squareform  (a)

% perform classical mds
[ya,e]=cmdscale  (b)

% truncate mds matrix to individual parts - sera of individuals
infected
% with different strains

sab=ya  (1:20,:)
ba=ya  (21:40,:)

%plot the graphs
figure
%sab infected children
```

```
plot  (sab  (:,1),sab  (:,2),'bo',
'MarkerEdgeColor','b','Markersize',12,'MarkerFaceColor','g')
%text  (sab  (:,1),sab  (:,2),'SAB')
hold on

%ba infected children
plot  (ba  (:,1),ba  (:,2),'bo',
'MarkerEdgeColor','b','Markersize',12,'MarkerFaceColor','r')
%text  (ba  (:,1),ba  (:,2),'BA')
grid on
legend  ('Contemporary BA strains', 'Contemporary Non-BA strains')
axis  ([-50 55 -20 28])

%
==========================================================================
====
% This code performs multi dimensional scaling analysis using
distance data
% obtained from MEGA using RSV A G gene sequences obtained from
infants
% infected with RSV A.
%==========================================================================
======

clear
clc
% change working directory
chdir 'C:\Documents and Settings\csande.KWTRP\Desktop\paper1\thesis
chapter 7\'

%load data
RSVA_data = textread  ('C:\Documents and
Settings\csande.KWTRP\Desktop\paper1\thesis chapter 7\distance matrix
- RSV As-matlab-pded.csv', '', 'delimiter', ',', 'emptyvalue', NaN)

%run classical mds
[ya,e]=cmdscale  (RSVA_data)

%truncate the mds matrix to strain components
Ken_A_2006=ya  (1:1,:)
```

```
RSVA_NP12=ya  (2:20,:)
A2=ya  (21:21,:)


%plot the data - 2d



%NP12 infant samples
plot  (RSVA_NP12  (:,1),RSVA_NP12  (:,2),'bo',
'MarkerEdgeColor','b','Markersize',12,'MarkerFaceColor','y')
%text  (SAB  (:,1),SAB  (:,2),'SAB')
hold on



%A2 test strain
plot  (A2  (:,1),A2  (:,2),'bo',
'MarkerEdgeColor','b','Markersize',12,'MarkerFaceColor','g')
%text  (A2  (:,1),A2  (:,2),'A2')
hold on

%Ken/A/2006 test strain
plot  (Ken_A_2006  (:,1),Ken_A_2006  (:,2),'bo',
'MarkerEdgeColor','b','Markersize',12,'MarkerFaceColor','b')
%text  (Ken_A_2006  (:,1),Ken_A_2006  (:,2),'Ken_A_2006')
hold on



legend  ('Contemporary RSV A strains','A2/A/1961','Ken/A/2006')
 grid on

axis  ([ -0.0105 0.035 -0.0065 0.0085])
```

STATA CODE:

```
. //=======================================================
```

```
. // Code: This code compares the magnitude of the serum neutralising
. //         response to A2 and Ken/A/2006 by infants infected with
. //         contemporary RSV A strains in the 2005/2006 RSV A epidemic
. //         in Kilifi
. //=======================================================
.
.
. capture: drop gid

. capture: label drop gid

. gen gid=.  // contains 1 if sera from RSV A infected kids was tested against A2
  (329 missing values generated)

.            // and 2 if it was tested agains Ken/A/2006
. label define gid 1 "A2  (Aus/A/1961)" 2 "Ken/A/2006"

. label values gid gid

. replace gid=1 if i_gp==1 & t_virus_desc==1 //A2
  (45 real changes made)

. replace gid=2 if i_gp==1 & t_virus_desc==2 // ken/a/2006
  (45 real changes made)

. xi: regress log10foldrise i.gid
i.gid             _Igid_1-2   (naturally coded; _Igid_1 omitted)

      Source |       SS       df       MS              Number of obs =      66
-------------+------------------------------           F  (  1,    64) =    0.10
       Model |  .020741024     1  .020741024           Prob > F      =  0.7564
    Residual |  13.6820939    64  .213782718           R-squared     =  0.0015
-------------+------------------------------           Adj R-squared = -0.0141
       Total |   13.702835    65  .210812846           Root MSE      =  .46237

------------------------------------------------------------------------------
log10foldr~e |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
     _Igid_2 |  -.0354546   .1138267    -0.31   0.756    -.2628497    .1919404
       _cons |   .5339482   .0804877     6.63   0.000     .3731556    .6947407
------------------------------------------------------------------------------

. di 10^.5339482  //fold rise to A2
3.4193866

. di 10^  (.5339482 -.0354546) //fold rise to ken/a/2006
3.1513279



. //=======================================================
. // This code compares antibody responses to the RSV A and B constant regions
```

```
. //of the G protein in acute and convalescent sera obtained from
. // infants with RSV A and B infections
. //============================================================
.
.
. // responses to constant region of RSV A G protein
. ttest    od_a_ag_acute ==od_a_ag_conv if i_gp==1 // acute/conv responses by RSV A
infecteds

Paired t test
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
od_a_a~e |      57    1.040544    .0907856    .6854161    .8586785    1.222409
od_a_a~v |      57    1.800292    .0893282    .6744131    1.621347    1.979238
---------+--------------------------------------------------------------------
    diff |      57   -.7597485    .1178534    .8897736   -.9958373   -.5236598
------------------------------------------------------------------------------
     mean  (diff) = mean  (od_a_ag_acute - od_a_ag_conv)              t =  -6.4466
 Ho: mean  (diff) = 0                                 degrees of freedom =       56

 Ha: mean  (diff) < 0           Ha: mean  (diff) != 0           Ha: mean  (diff) > 0
 Pr  (T < t) = 0.0000         Pr  (|T| > |t|) = 0.0000          Pr  (T > t) = 1.0000

. ttest    od_a_ag_acute ==od_a_ag_conv if i_gp==2 // acute/conv responses by RSV B
infecteds

Paired t test
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
od_a_a~e |      34    1.226078    .0798425    .4655581    1.063638    1.388519
od_a_a~v |      34    1.242216    .1139764    .6645908    1.010329    1.474102
---------+--------------------------------------------------------------------
    diff |      34   -.0161372     .128561    .7496328   -.2776965     .245422
------------------------------------------------------------------------------
     mean  (diff) = mean  (od_a_ag_acute - od_a_ag_conv)              t =  -0.1255
 Ho: mean  (diff) = 0                                 degrees of freedom =       33

 Ha: mean  (diff) < 0           Ha: mean  (diff) != 0           Ha: mean  (diff) > 0
 Pr  (T < t) = 0.4504         Pr  (|T| > |t|) = 0.9009          Pr  (T > t) = 0.5496

.
. //responses to constant region of B G protein
. ttest    od_b_ag_acute ==od_b_ag_conv if i_gp==1 // acute/conv responses by RSV A
infecteds

Paired t test
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
```

```
od_b_a~e |      57    1.639544     .107474     .8114109    1.424248     1.85484
od_b_a~v |      57    1.692901     .121133     .9145344    1.450242    1.935559
---------+--------------------------------------------------------------------
    diff |      57   -.0533567     .0895749     .6762757   -.2327968    .1260834
------------------------------------------------------------------------------
     mean  (diff) = mean  (od_b_ag_acute - od_b_ag_conv)              t =  -0.5957
 Ho: mean  (diff) = 0                                     degrees of freedom =       56

 Ha: mean  (diff) < 0           Ha: mean  (diff) != 0           Ha: mean  (diff) > 0
 Pr  (T < t) = 0.2769         Pr  (|T| > |t|) = 0.5538          Pr  (T > t) = 0.7231

. ttest    od_b_ag_acute ==od_b_ag_conv if i_gp==2 // acute/conv responses by RSV B
infecteds

Paired t test
------------------------------------------------------------------------------
Variable |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
od_b_a~e |      34    1.389804    .1284202    .7488118    1.128531    1.651077
od_b_a~v |      34    3.153794    .1052459    .6136835     2.93967    3.367918
---------+--------------------------------------------------------------------
    diff |      34    -1.76399    .1717826    1.001656   -2.113485   -1.414496
------------------------------------------------------------------------------
     mean  (diff) = mean  (od_b_ag_acute - od_b_ag_conv)              t = -10.2687
 Ho: mean  (diff) = 0                                     degrees of freedom =       33

 Ha: mean  (diff) < 0           Ha: mean  (diff) != 0           Ha: mean  (diff) > 0
 Pr  (T < t) = 0.0000         Pr  (|T| > |t|) = 0.0000          Pr  (T > t) = 1.0000

.
. //comparison of seroconversion rates
. tab aserocon i_gp,chi2 //seroconversion rates to RSV A constant region by RSV A/B
infected
> s

           |         i_gp
  aserocon |         A          B |     Total
-----------+----------------------+----------
        No |        41         34 |        75
       Yes |        16          0 |        16
-----------+----------------------+----------
     Total |        57         34 |        91

          Pearson chi2  (1) =  11.5799   Pr = 0.001

. tab bserocon i_gp,chi2 //seroconversion rates to RSV B constant region by RSV A/B
infected
> s

           |         i_gp
  bserocon |         A          B |     Total
```

No	57	27	84
Yes	0	7	7
Total	57	34	91

Pearson chi2 (1) = 12.7132 Pr = 0.000